THE ROLE OF CLAUDIN-1 IN INFLAMMATION AND COLON CANCER

By

Jillian L. Pope

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

May, 2014

Nashville, Tennessee

Approved:

Dr. Albert B. Reynolds

Dr. R. Daniel Beauchamp

Dr. Anna L. Means

Dr. Punita Dhawan

ORIGINAL PUBLICATION

Pope, J.L., Bhat, A.A., Sharma, A., Ahmad, R., Krishnan, M., Washington, M.K., Beauchamp, R.D., Singh, A.B., Dhawan, P., 2013. Claudin-1 regulates intestinal epithelial homeostasis through the modulation of Notch-signalling. Gut. Published Online First: 13 June 2013. *doi:10.1136/gutjnl-2012-304241*

DEDICATION

To my family, for all their love and support through this journey,

may this be a source of inspiration to persevere and achieve your goals;

To my cousin Carissa, who makes the implications of this research real for me,

I admire your strength in living with colitis;

&

In loving memory of my mother, Rebecca Pope, my best friend, role model, and

biggest supporter, I know you would be proud

ACKNOWLEDGEMENTS

This work would not have been possible without the financial support that I have received to perform my research. The work in this dissertation was performed in the Department of Cancer Biology at Vanderbilt University from 2008-2014. This work was supported by the following: NIH grant CA124977 (P. Dhawan), DK088902 (A.B. Singh), VICC P30CA68485, and DDRC DK58404. Additionally I would like to acknowledge additional funding in support of my training: MITP training grant 5T32 CA009592-23, supplement, and The Initiative for Maximizing Student Diversity (IMSD) grant -NIH 5R25GM062459.

I am truly grateful for all those who have supported me and prayed for me during my journey. The graduate student experience is riddled with uncertainties that include a series of highs and lows, which I am sure have helped shape me into becoming scientist I am today. It would not have been possible to navigate this experience on my own, and I am grateful to have had wonderful people to support me.

I would first like to thank my advisor, Dr. Punita Dhawan, for accepting me into her lab and supporting me throughout the duration of my graduate career. Her time and patience throughout my studies, as well as during one of the hardest personal moments of my life, is well appreciated. I have learned much as a scientist in this lab, even beyond technical experience, and am sure that all I have learned has provided me a firm foundation to build upon as I continue my career. It has been an interesting experience watching the growth and

development of the lab and, even more so, the progression of my research. Additionally, I would like to thank everyone in the Dhawan and Singh Labs, including current and past lab members. It is rare that you encounter people from such different backgrounds yet be able to form a wonderful working relationship and friendship. It is especially rare in science that you can work among a group of people who genuinely wish for you to succeed and who do all they can to make that happen. I would like to especially thank the past and present postdocs; Dr. Moorthy Krishnan, Dr. Ajaz Bhat, Dr. Rizwan Ahmad, and Dr. Ashok Sharma. I wish each of you continued success in your careers and appreciate the role that each of you contributed to mine.

Secondly I would like to thank my committee members. It was such a wonderful feeling to move past a sense of “nervousness” in going before my committee to realizing that they were actually a great team of support, whose purpose was to stretch me beyond my self-perceived capabilities. You may not realize it, but the conversations I’ve shared with each of you has helped me at some point in my graduate career to regain my confidence and to continue to push forward. Additionally, I am glad I have gotten the opportunity to observe how each of you think and approach scientific research. To my chair, Dr. Al Reynolds, I appreciate the talks of support and encouragement, and for helping me to realize I needed to relocate my “bug for science.” I am grateful for your refreshing perspective and have always appreciated you making the time to meet with me. To Dr. Beauchamp, I thank you so much for your time and service to my committee. Although the conversations were brief, you’ve helped me to think big

and to aim high as I further my research career. Dr. Anna Means, I am super grateful for your service to my committee. You've really helped me to focus on the science and to think of the totality of my work. I especially value your honesty, encouragement, and the fact that you pushed me to be more vocal and to take charge, to be an active contributor in science.

The advisor and committee members are only a fraction of the people that one interacts with, especially in a collaborative environment as the one that exists at Vanderbilt. I would like to thank each of the members and staff of the Beauchamp lab, which I have always felt was my second lab "home". I would like to especially thank my colleague and friend Tanner Freeman (MD-PhD candidate in Beauchamp lab). Your assistance and support have always been appreciated and I thank you tremendously. Additionally, I would like to thank fellow Cancer Biology colleagues Sarah Short (PhD candidate in Reynolds lab), Dr. Caitlyn Barrett and Shenika Poindexter (PhD candidate in Williams Lab), and Andreia Bates (PhD Candidate in Fingleton Lab). Each of you have been an invaluable aid and resource to me throughout my studies. I only hope that I have been as much help to you as you were to me.

Special thanks to the staff of the Department of Surgery and the Department of Cancer Biology, including former staff member Tracy Tveit. Additionally I would like to thank current and former staff members of the BRET office, especially Ms. Cathleen Williams. A heartfelt thanks to Dr. Roger Chalkley and Dr. Linda Sealy for their genuine concern for students' success and well-

being. You have been a great support to my graduate career from beginning to end and I hope you know that you are appreciated by many.

Last but not least, I would like to thank my family and friends. To my Vanderbilt friends/colleagues, I am grateful to have met each of you and I am glad that I was able to experience this journey with you. To my long-time friends, I thank you all for your continued support throughout my studies. To my family, I am truly thankful for your prayers, encouragement and your counsel during my studies. Although you may not have completely understood what I have been doing, I appreciate your total support, and most of all, for believing in me.

I would especially like to thank my grandparents, aunts and uncles who immediately stepped up to support me since the loss of my mother. Although no person can fill her void, I appreciate all that you have done to make the hole feel less empty. To my father (James), Kevin, Jessica, Chad, Tekia, and KJ: I love you and I thank you for everything!

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

APC – adenomatous polyposis coli

CD – Chron's Disease

cDNA – complementary DNA

Cl-1 Tg – Claudin-1 Transgenic mice

CRC – colorectal cancer

DNA – Deoxyribonucleic Acid

EMT – Epithelial to mesenchymal transition

FAP – Familial adenomatous polyposis coli

IACUC – institutional animal care and use committee

IBD – Inflammatory bowel disease

IEC – Intestinal epithelial cell

IHC – Immunohistochemistry

IL-1β – Interleukin 1, beta

kDa – kilodalton KLF4 – Kruppel like factor 4

MMP9 – Matrix metalloprotease 9

mRNA – messenger RNA (Ribonucleic Acid)

Muc2 – Mucin 2

PAS – Per Acidic Schiff's

qRT-PCR – Quantitative real time – polymerase chain reaction

RNA – Ribonucleic Acid

SI – Small intestine

TER – Transepithelial resistance

TNFα – Tumor Necrosis Factor, alpha

TFF3 – Trefoil factor 3

WT – Wild Type

CHAPTER I

INTRODUCTION

Perturbation of physiological processes stimulate a cascade of events resulting in adverse pathogical conditions, the most predominant being cancer. Colorectal cancer (CRC) is the third most common cancer in the United States and the second leading cause of cancer-related deaths. CRC is most commonly initiated by loss of function mutations of *Adenomatous polyposis coli* (*APC*), a tumor suppressor gene of the Wnt/B-catenin pathway, which leads to constitutive activation of Wnt signaling. Although significant progress has been made in understanding the sequential genetic events leading to cancer development, the precise genes and associated molecular events underlying tumor progression are still poorly understood. Our lab has previously reported a correlation between increased expression of claudin-1, a tight junction protein, in primary and metastatic tumor samples of CRC patients (Dhawan et al., 2005). Additionally, in adenomas of the APC^{Min} mouse, an experimental model for CRC, and human cell lines harboring mutations in APC, claudin-1 expression is highly increased and mislocalized. Taken together, these studies suggest existence of a potential interplay between dysregulated claudin-1 expression and APC, thus supporting an essential role for claudin-1 in the regulation of sporadic colonic neoplasia. In addition to genetic stimuli, CRC can be initiated by an inflammatory stimulus.

Patients with inflammatory bowel disease (IBD), a chronic disorder of the gastrointestinal tract, are at high risk for developing CRC. This may be attributed, in part, to repeated injury and repair, which induces transformation of the colonic epithelium. As such, increased Claudin-1 expression is also reported to be associated with active IBD and colitis-associated cancer, yet its distinct role in inflammation has yet to be elucidated. The purpose of this study is to identify the specific contribution that increased expression of a tight junction protein, claudin-1, brings to inflammation and CRC.

Tight Junctions

Cellular adhesion molecules are an important aspect of cell biology as they are essential to the formation of close contacts between adjacent cells. In the case of epithelial cells, these cell-cell contacts facilitate the formation of epithelial sheets, which constitute the surface and lining of organs throughout the body. The cellular adhesion molecules are composed of desmosomes, gap junctions, adherens junctions and the tight junctions. The tight junctions are the most apical of the cell-cell contacts, located at the “kissing” point between cells. They play major roles in regulating cellular polarity and paracellular permeability. In the context of cellular polarity, tight junctions serve as a molecular “fence” helping to maintain the segregation of apical and basolateral proteins (Shin et al., 2006). More studied is their barrier function, regulating cellular permeability, in which tight junctions serve as molecular “bouncers”, permitting or restricting the passage of ions, nutrients, and luminal antigens across the epithelium (Balda et al., 1996; Schmitz et al., 1999).

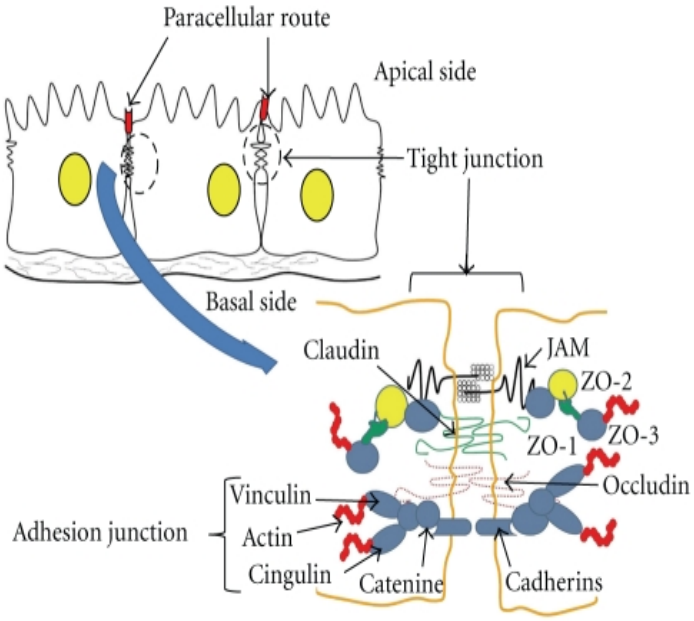


Figure 1. Schematic of Tight Junctions

The tight junctions reside at the most apical region of cellular junctions. They consist of claudins, JAMs, and occludin proteins. Peripheral tight junction proteins, ZO family, bind to membrane spanning junctions, and serve as connection to the actin cytoskeleton.

The tight junctions are composed of transmembrane spanning proteins and peripheral proteins, which rest near the surface of the cytosolic membrane (Figure 1). The most common peripheral tight junctions include those of the zona occludens family, ZO-1, -2, and -3 (Shin et al., 2006). The transmembrane proteins consist of three major families: junctional adhesion molecules (JAMs), occludin, and claudins. The claudin family is made of approximately 27 members with molecular weights ranging between 20-27 kDa. These proteins are made of four transmembrane domains, with their N- and C- terminal ends located in the cytoplasm. The C-terminus contains a binding site for the interaction with other proteins, including those containing PDZ binding domains that serve as a direct connection to the actin-cytoskeleton, and also contains residues that serve as sites for post-translational modifications. The claudins are expressed ubiquitously throughout various organs of the body facilitating cell-cell connections among epithelial and endothelial cells and have the ability to form homodimers and heterodimers. These interactions and varied expression patterns help confer special properties to the function of the organ. For example, claudin-7 has been shown to regulate paracellular conductance of sodium ions in kidney cells (Alexandre et al., 2005), and claudin-2 can regulate permeability and cation selectivity in the kidney (Muto et al., 2010). Claudin-1 plays a critical role in regulating barrier function in the skin and kidney (Tsukita and Furuse, 1998), (Inai et al., 1999).

Claudin-1 was the first of the claudins to be identified, alongside Claudin-2, in key experiments demonstrating that apical junctions were still able to form

during development, even in the absence of occludin, which was thought to be the sole integral membrane tight junction at the time (Furuse, 1998). Subsequent studies further demonstrated the importance of claudin-1 in regulating barrier function whereby overexpression of claudin-1 was able to increase transepithelial resistance (TER) and decrease paracellular permeability (Inai et al., 1999). The physiologic importance of claudin-1 was made even more evident by the generation of the Claudin-1 knockout mouse. These mice died within one day of birth due to epithelial barrier defects and dehydration, which showed its necessity for normal barrier function (Furuse et al., 2002). As indicated above, tight junctions, claudins in particular, are essential to the integrity of the epithelium and the organisms as a whole. Disruption of these cellular contacts can upset homeostatic interactions that prove detrimental to the organism. The intestine is one organ that depends heavily upon the function of the tight junctions.

Colonic homeostasis

The colonic epithelium exists in a delicate balance between millions of bacteria and antigens in the lumen and the immune regulating cells that reside in the lamina propia. Claudins help to regulate this balance by maintaining cellular polarity and barrier dynamics.

Another important aspect of epithelial homeostasis is the mucous barrier. The mucosal barrier aids in the luminal defense against environmental factors, bacterial invasion, and protection of the epithelium against injury (Hasnain et al., 2010), (McGuckin et al., 2011), (Hollingsworth and Swanson, 2004), (Kufe, 2009). Second to the tight junction, it helps maintain integrity of the epithelial

barrier. The primary constituents of the mucosal barrier are a family of glycoproteins termed Mucins. The primary secreted mucin, Muc2, has been extensively implicated in IBD and colorectal cancer. Muc2 deficient mice develop colitis, spontaneously, and enhance the development of colon cancer suggesting an essential role of Muc2 in epithelial homeostasis and disease progression (van der Sluis et al., 2006), (K. Yang et al., 2008). Mucins are secreted by specialized intestinal goblet cells and assemble to form the mucosal barrier that overlays the epithelium. This introduces yet another level of homeostatic control involving the colonic epithelium itself.

Differentiation of the colonic epithelium is tightly regulated by molecular cues at play in the colonic crypts. These cues predominately arise from the Wnt and Notch pathways. Active Wnt signaling is concentrated to the base of the crypt in rapidly proliferating cells, helping to maintain the stem cell population. Along the crypt axis, from the base of the crypt upwards, Wnt cues gradually taper off, regulating expression of genes within the epithelial cell that leads to a state of terminal differentiation. The stem cells of the crypt give rise to transit-amplifying cells. It is after this stage that Notch signaling determines the cell fate between the secretory and absorptive cell lineages. The absorptive lineage gives rise to terminally differentiated colonocytes. The secretory lineage produces hormone secreting enteroendocrine cells and the mucous secreting goblet cells.

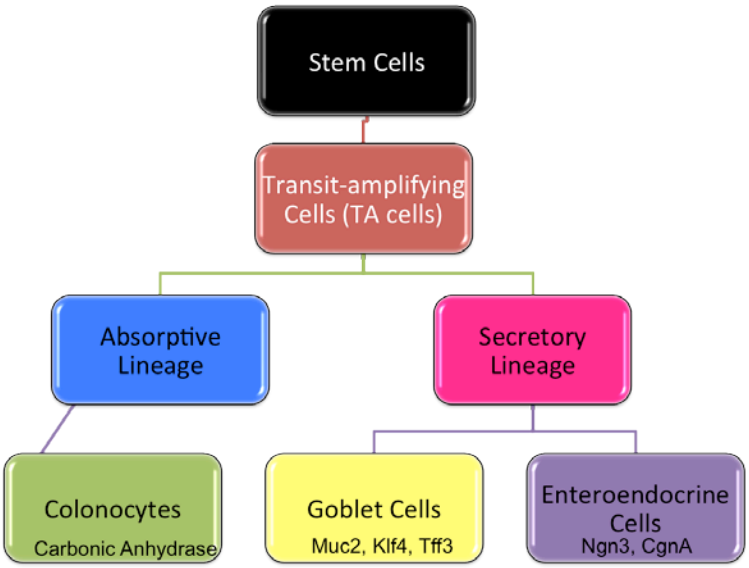


Figure 2. Epithelial Differentiation of the Colon

The stem cells of the colonic crypt give rise to transit-amplifying cells. These cells give rise to cell types of the absorptive lineage or the secretory lineage. Absorptive cells of the colon are colonocytes, identified by the marker carbonic anhydrase. Cells of the secretory lineage produce mucous secreting goblet cells that are characterized by Muc2, Klf4 and Tff3 gene expression. Enteroendocrine cells are secretory cells identified by expression of neurogenin 3 (Ngn3), and Chromagranin A (CgnA).

Notch activation is initiated by the interaction of the NOTCH ligand (Delta-like ligand -1, -3, or -4 and JAG1, JAG2) with a NOTCH receptor (NOTCH 1-4) of an adjacent cell. This interaction facilitates a series of proteolytic cleavages that results in the release of the Notch Intracellular Domain (NICD) to translocate to the nuclease and bind its transcriptional cofactor, CSL. This complex will bind DNA and activate transcription of the HES/HEY family of transcriptional repressors. Hes1 in turn inhibits expression of Math1. Math1 is a gene that is responsible for the transcription of Mucin2, the primary constituent of the mucous secreting goblet cells. Several components of the Notch signaling have been shown to be upregulated in CRC (Reedijk et al., 2008). More recent studies have investigated targeting Notch pathway components as a strategy to inhibit colon tumorigenesis (Miyamoto et al., 2013), (Sonoshita et al., 2011). Additionally, the Notch pathway has been implicated in inflammatory bowel disease as being crucial to regeneration of colonic epithelium after injury (Okamoto et al., 2009).

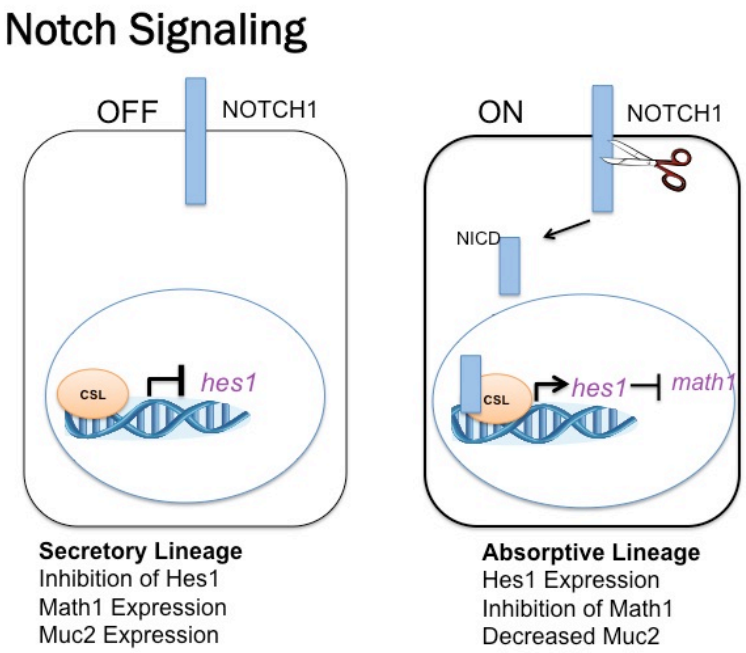


Figure 3. Notch Signaling Overview

During epithelial differentiation, when Notch signaling is in the "OFF" state Hes1 expression is repressed and cells continue to express Math1, which give rise to specialized secretory cells. When Notch signaling is activated to the "ON" state, a series of proteolytic cleavages release Notch Intracellular Domain (NICD), facilitating translocation to the nucleus and binding to CSL. This activates the Hes1 transcriptional repressor to inhibit Math1 expression, causing cells to give rise to the absorptive lineage.

Claudins in disease

Defective barrier function has been shown to result in a myriad of diseases including inflammatory bowel disease (Laukoetter et al., 2008), atopic dermatitis (Hogan et al., 2012), deafness (Gow et al., 2004), (Ben-Yosef et al., 2003), and jaundice of the liver including primary biliary cirrhosis and primary sclerosing cholangitis (Sawada, 2013). Based on the function of claudins and tight junctions in colonic homeostasis, it can be appreciated how their dysregulation can result in adverse effects to the epithelium, the organ in which it protects, and the organism as a whole. Additionally, dysregulated junctions can also lead to a loss in polarity, creating an EMT-like condition, that induces de-differentiation and transformation of cells that can lead to accelerated tumor growth and progression (Singh et al., 2010). Claudins are able to regulate each of these processes in addition to regulating proliferation, differentiation (Tsukita et al., 2008),(Pope et al., 2013), migration (Ikari et al., 2011), and invasion (Oliveira and Morgado-Diaz, 2007), (Morin, 2005). Their differential expression in various cancer models has been shown to correlate with disease severity, and in some cases, advanced staging of tumor progression. Increased expression of Claudin-1, in particular, has been associated with disease severity in intestinal inflammation and colon cancer.

Intestinal Inflammation

Breeches in barrier dynamics can result in a shift in homeostasis of the affected tissue that can lead to inflammation, as is the case in the colon and the development of inflammatory bowel disease (IBD). Inactivity of the tight junction

associated protein Myosin-light chain Kinase (MLCK) was sufficient to alter immune regulation and enhance susceptibility to colitis (Su et al., 2009). Additionally, genetic depletion of claudin-7 in the intestine resulted in increased inflammation and epithelial sloughing, reminiscent of symptoms associated with IBD (Ding et al., 2012). IBD is a broad classification for a group of chronic inflammatory disorders affecting the gastrointestinal tract, the most common being Chron's Disease and Ulcerative Colitis. Although colitis can be inherited, this is only the case for a small percentage (less than 10%) of affected individuals. As such, the majority of patients acquire the disease spontaneously. Since the exact etiology of IBD is unknown, there is no known cure. Some therapies, that target the inflammation, are employed to temporarily alleviate active disease. Most patients become accustomed to management of the disease through diet, yet even this does not prevent recurring inflammation. Many patients will develop this disease as young adults and may suffer years of successive rounds of inflammation and epithelial repair, which puts them at high risk for developing colorectal cancer. There have been many studies undertaken to identify the initiating stimulus of colitis, and most have resulted in adding significant knowledge to the field. However, the vast number of studies have only highlighted the complexity of this disease. Genetic mutation, environmental stimuli, dysregulation of the immune system, and contribution of the commensal bacteria are all factors that have been investigated and suggested to play a role, singularly or in concert, in the development of IBD.

Surprisingly, there are few studies that show claudin deficiency leading to spontaneous disease. Some, however, have identified their dysregulation during disease. Increased expression of claudin-1 and claudin-2 was observed in active inflammatory bowel disease of human patient samples (Weber et al., 2008), and changes in Claudin-5 and -8 expression have been associated with Chron's disease (Zeissig et al., 2007). In an attempt to reconcile these changes, studies have shown that inflammatory components such as TNFa, IL-6 and IFNy can regulate claudin expression (T. Suzuki et al., 2011), (Shiozaki et al., 2012), (Tedelind et al., 2003), suggesting a potential mechanism for altered expression of tight junctions in IBD.

Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer in the United States. CRC can be initiated by an inflammatory stimulus, known as colitis-associated cancer, or can be acquired spontaneously, as is the case in over 75% of the total CRC cases. Over 90% of sporadic colorectal cancer cases occur in patients having a hereditary disease known as Familial Adenomatous polyposis (FAP), in which only one copy of the APC allele is inherited. The APC protein is a key player in the Wnt signaling pathway. It assembles with other proteins to make up the b-catenin destruction complex, which is responsible for the continued degradation of b-catenin in the absence of Wnt ligand. Upon Wnt stimulation, the APC complex disassembles, facilitating accumulation of b-catenin and interaction with transcription cofactors TCF/LEF to activate expression of genes that regulate cell cycle dynamics. Extensive research has

shown that the constitutive activation of the Wnt signaling pathway, either by loss of APC or activating mutations in b-catenin, is most frequently associated with development of colorectal cancer.

Claudin-1 has been identified as a target gene of Wnt signaling. Its promoter contains two putative TCF/LEF binding sites to which β-catenin can bind and activate transcription (Miwa et al., 2001). To this end, increased claudin-1 expression has been frequently observed in colorectal cancer (Resnick et al., 2005), (Huo et al., 2009). Primary tumor samples obtained from human patients exhibit elevated claudin-1 protein expression compared to adjacent normal tissue (Dhawan et al., 2005). Claudin-1 expression was further upregulated in metastatic tissue samples obtained from the same patients. Additionally, in APC^{Min} mice, claudin-1 expression is elevated in intestinal adenomas compared to adjacent normal tissue. Even more surprising, genetic modulation of claudin-1 in human colon cancer cells was sufficient to regulate primary tumor growth and metastasis in mouse xenograft studies.

Dysregulated claudin-1 expression is not only restricted to spontaneous cancers. Analysis of patient samples showed increased expression of claudin-1 in colitis-associated CRC(Kinugasa et al., 2010). This type of CRC is acquired by patients who endure years of chronic inflammatory disease, which eventually leads to transformation of the colonic epithelium. Indeed, claudin-1 expression is upregulated in patients with chronic inflammatory disease, and more interestingly in areas of dysplasia (Weber et al., 2008). As such, understanding the role of

tight junctions, and specifically that of claudin-1, in inflammation will also aid in a better understanding of its role in CRC.

Purpose/Significance

Developing successful therapeutic strategies to prevent and combat colorectal cancer requires a better understanding of the processes that leads to tumor progression. Claudin-1 expression is upregulated in CRC and has been shown to regulate tumorigenic potential of CRC cells. Additionally, it is upregulated in colitis-associated cancer, and correlates with active IBD. The purpose of this study is to identify the specific contribution that increased claudin-1 expression brings to CRC. *Thus, our hypothesis is that claudin-1 overexpression contributes to the colon carcinogenesis when combined with a CRC initiating genetic mutation such as APC mutation.* Additionally, as inflammation can be a precursor to CRC development, *we predict increased expression of claudin-1 will also contribute to inflammation*. These studies will impact the current understanding of the role of tight junction proteins in the regulation of sporadic CRC especially in collaboration/association with APC mutation and inflammatory disease. Most importantly, this work will help to seek potentially new therapeutic opportunities.

CHAPTER II

CLAUDIN-1 REGULATES INTESTINAL EPITHELIAL HOMEOSTASIS THROUGH THE MODULATION OF NOTCH SIGNALING

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 (Turner, 2009). 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 (Chichlowski and Hale, 2008).

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 (Kufe, 2009). Tight junctions, the most apical cell-cell adhesions, are the primary regulators of the epithelial barrier function (Singh et al., 2010). Indeed, modulation of the muc-2 expression, goblet cell number and tight junction (TJ) integral proteins are known characteristics of IBD patients (Maloy and Powrie, 2011). Furthermore, mice deficient in muc-2 protein demonstrate an elevated production of pro-

inflammatory cytokines and develop colitis spontaneously (van der Sluis et al., 2006). 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 (Dahan et al., 2011; Qiao and Wong, 2009; Sikandar et al., 2010; Zheng et al., 2011).

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 (Singh et al., 2010). Notably, expression of the claudin-1 protein is increased in the areas of active inflammation (Weber et al., 2008). Of interest, a correlation between increased claudin-1 expression and neoplastic transformation was also noted in colitis-associated cancer (Kinugasa et al., 2010). Also, loss of claudin-1 leads to severe dehydration and post-natal death in mice (Furuse et al., 2002). We have reported a causal association of claudin-1 expression with sporadic colon cancer growth and progression (Dhawan et al., 2005). However, the role of claudin-1 in the regulation of epithelial homeostasis, mucosal inflammation and IBD remains unknown.

In the current study, using mice with intestinal specific overexpression of Claudin-1 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 (Marjou et al., 2004). 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 1).

Induction of Colitis by Dextran Sodium Sulfate

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 2). 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 0.05 were considered significant.

Cell Culture and Transfection

The human colon cancer cell lines, SW480 and LS174T were obtained from ATCC and cultured in RPMI containing 10% FBS. $SW480^{Claudin\text{-}1}$ cells have been described previously (Dhawan et al., 2005). Stable polyclonal cells, $LS174T^{Claudin\text{-}1}$ were obtained in the same manner. Stable cell lines were maintained using neomycin as the selection reagent. For inhibitor treatments, cells were seeded in six-well cell culture plates to final density of 60% confluence before adding indicated amounts of inhibitor for the indicated time points.

Experimental Colitis

During DSS treatment, mice were monitored daily for signs of distress including, excessive loss of weight (20%), hunched posture, and bloody stool. Mice reaching critical weight loss were sacrificed according to IACUC guidelines.

Tissue Harvesting

Following sacrifice, the small intestine and colon were dissected, flushed with PBS, opened flat, and formalin-fixed via the Swiss roll method. Samples were further processed by the Vanderbilt Translational Pathology Shared Resource Core. Distal and proximal ends of the colon were snap-frozen in liquid nitrogen. Other tissues harvested for protein expression were also processed in this manner. All frozen tissues were stored at -80ºC until further analysis.

Immunostaining

Paraffin-embedded tissues were de-pariffinized with xylene and successive ethanol washes. Antigen unmasking was performed using 10mM sodium citrate buffer (pH 6.0). Immunostaining was performed using the indicated antibodies (Table 1) and VectaStain ABC kit (Vector Laboratories) was used for brown staining. FITC and Rhodamine conjugated antibodies against mouse and rabbit antibodies (Jackson laboratories) were used for immunofluorescence staining. Images were obtained using a Zeiss light microscope or Confocal microscopy (LSM 510) in part through the use of the VUMC Cell Imaging Shared Resource. For PAS immunostaining, cells were plated on glass chamber slides and treated as mentioned previously. Upon termination of treatment, cells were fixed for 15 min and room temperature with

ethanol-acetic acid (3:1), then stained with periodic acid solution for 5 min at room temperature. Slides were then washed with tap water to remove excess stain and incubated with Schiffs reagent for 15 min at room temperature. After washing with tap water for 5 min, cells were counterstained with hematoxylin.

RNA Isolation for quantitative RT-PCR

Total RNA from mouse samples was extracted using 20-30mg of tissue from the distal colon using the RNeasy Mini Kit (Qiagen) with DNAse digestion. RNA integrity was determined by formaldehyde gel electrophoresis and absorbance spectroscopy. Samples presenting two bands, corresponding to the 18S and 28S subunits, and having an A260/A280 of ~1.8 were used for experiments.

Quantitative RT-PCR

Total RNA (1ug) of each sample was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). PCR reactions were performed using gene-specific primers (Table 2). Each well contained SYBR Green Master Mix (Qiagen), primer sets and cDNA (30ng). Samples were loaded in triplicates or quadruplicates in 96 well plates and run on a Bio-Rad iCycler. Ct values were utilized to calculate fold change. Normalization was performed using beta-actin.

Immunoblot analysis

Cells were lysed in radioimmunoprecipitation assay buffer, and Western blotting was performed as previously described (Dhawan et al., 2005). Antibody information can be obtained in Table 1.

In vivo Permeability Assay

Mice were administered 4 kDa FITC-dextran via enema to measure permeability. Mice were anesthetized and blood taken at baseline and thirty minutes following rectal administration from the retro-orbital sinus of the eye. Serum was collected from the blood via centrifugation. Samples were measured for fluorescence using a microplate fluorescence reader (BIO-TEK) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The concentration was determined using a standard curve.

Ussing Chamber Studies

Distal colon segments were harvested from mice and mounted in Ussing chambers (Physiologic Instruments, Inc., San Diego, CA). Samples were mounted between the two halves the Ussing chamber (0.1-cm^2 opening). The mucosal and serosal sides of the chambers were filled with 5mL of Ringer's solution (150 mM NaCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, 10mM mannitol, 10mM glucose, and 10mM Tris-HEPES, pH 7.4). The ringer solution was continuously oxygenated with 100% O_2 bubbled through solution. After allowing 20 minute equilibration period to measure baseline potential difference and resistance, 4kDa FITC Dextran (Sigma) was added to the mucosal compartment. Two-hundred microliter samples were taken every 15 minutes from the serosal compartment for analysis and replaced by fresh Ringer solution. Permeability was determined at the end of the 120-min experimental period and assessed for fluorescence in a microplate fluorescence reader (BIO-TEK).

Electron Microscopy

Colon tissue from WT and Tg mice were processed and photomicrographs were obtained/analyzed through the assistance of the VUMC Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, HD15052, DK59637 and EY08126).

Table 1. Antibodies and Dilutions

Antibodies	Company	Western Blot	IHC
Claudin-1	Invitrogen	1:1000	1:200
Claudin-2	Invitrogen	1:1000	1:200
Claudin-3	Invitrogen	1:1000	1:200
Claudin-4	Invitrogen	1:1000	1:200
Claudin-7	Invitrogen	1:1000	1:200
Claudin-15	Invitrogen	1:1000	1:200
Occludin	Invitrogen	1:1000	1:200
B-catenin	BD Biosciences	1:3000	1:300
E-cadherin	BD Biosciences	1:3000	1:300
Mucin-2	Santa Cruz		1:400
Carbonic Anhydrase			1:400
phospo-ERK	Santa Cruz	1:500	
NICD	Hybridoma	1:500	
MMP9	Abcam	1:1000	
Hes1	Santa Cruz		1:200

Table 2. Primers

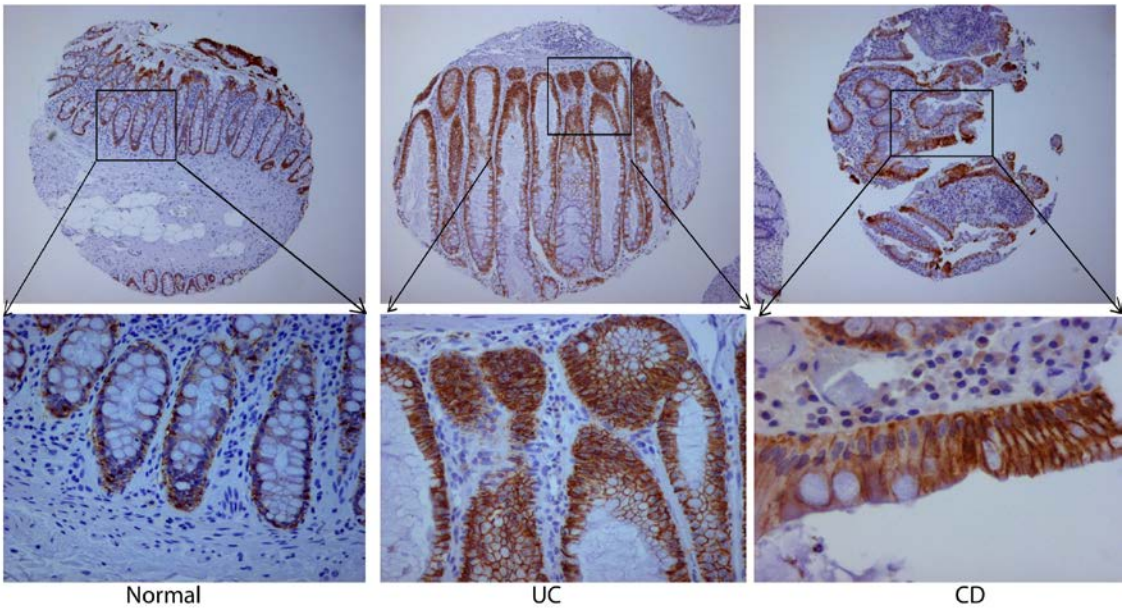
Primer Sequences		
	FORWARD	**REVERSE**
Genotype A	GGC GCC CTT AAG ATA CAT TG	GCC AGT TTC CCT TCT TCC TC
Genotype B	TGG CGC CCT TAA GAT ACA TT	GGT CAG GCT CTC TTC ACT GG
Real Time		
mHes1	AAA ATT CCT CCT CCC CGG TG	TTT GGT TTG TCC GGT GTC G
mMath1	AGC TGT CCA AAT ATG AGA CCC TAC A	GAC ATT GGG AGT CTG CAG CAA
hHES1	ACA CGA CAC CGG ATA AAC CAA	GCC GCG AGC TAT CTT TCT TCA
hMATH1/atoh1	CCT TCC AGC AAA CAG GTG AAT	TTG TTG AAC GAC GGG ATA ACA T
hACTIN	CCA AGC ACA ATG AAG ATC AA	ACA TCT GCT GGA AGG TGG AC
mTNFα	CTG TGA AGG GAA TGG GTG TT	GGT CAC TGT CCC AGC ATC TT
mIFNγ	GCC ACG GCA CAG TCA TTG AA	CGC CTT GCT GTT GCT GAA GA
mKC	GCT GGG ATT CAC CTC AAG AA	CTT GGG GAC ACC TTT TAG CA
mIL-10	TAG AGC TGC GGA CTG CCT TC	CTT CAC CTG CTC CAC TGC CT
mACTIN	CCA GAG CAA GAG AGG TAT CC	CTG TGG TGG TGA AGC TGT AG

Results

Characterization of the Cl-1Tg mice

Claudin-1 expression is upregulated under multiple intestinal pathological conditions including IBD and colorectal cancer (Dhawan et al., 2005; Kinugasa et al., 2010; Weber et al., 2008). We further confirmed increased claudin-1 expression in Crohn's disease and Ulcerative colitis patient samples (Figure 4).

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 5A). As predicted, robust claudin-1 overexpression was observed in the colon, small intestine, and cecum of the transgenic mice (Figure 5B), but not in other organs (Figure 6A). 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 5C).



	Claudin-1	Expression	
	Low	Medium	High
Normal	40% (2)	60% (3)	0
UC	0	60% (3)	40% (2)
CD	0	50% (3)	50%(3)

Figure 4. Representative patient samples from normal, Ulcerative Colitis, and Chron's Disease

Representative patient samples from normal, Ulcerative colitis and Crohn's disease patient. IHC staining for claudin-1 was performed using archived paraffin sections of normal controls, Crohn's disease (CD) and ulcerative colitis (UC) (n=5-6) and quantitation depicted in the table as % samples (number of samples). Interestingly, increased claudin-1 immunostaining either appeared membrane bound (though it was localized rather laterally between the epithelial cells) or membrane localization was disrupted and appeared punctate or cytosolic and possibly correlated with the areas of active inflammation.

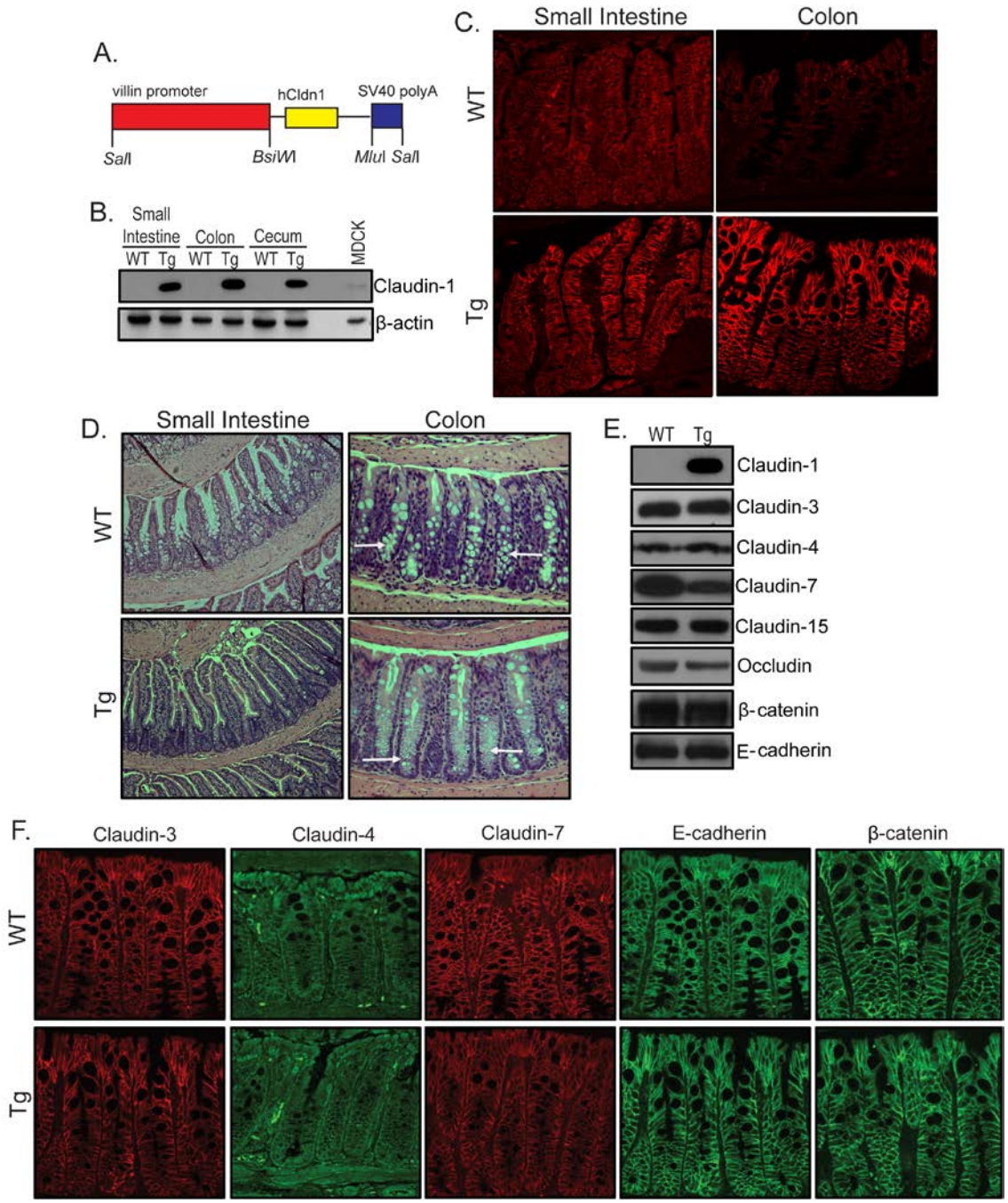


Figure 5. Generation of Claudin-1 Transgenic mice

(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.

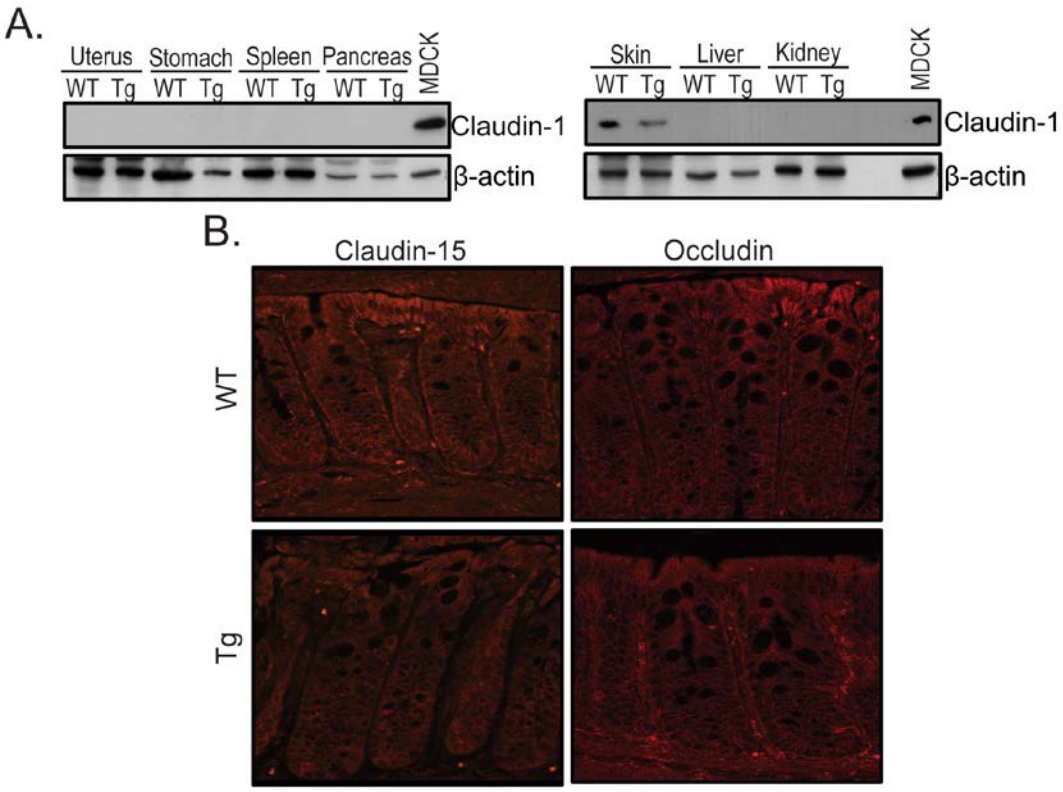


Figure 6. Expression analysis of Cl-1Tg mice

(A) Tissue lysates from indicated organs of wild type (WT) and Cldn-1TG (Tg) mice were tested for Claudin-1 expression. Actin was used as a loading control and MDCK cell lysate was used as positive control. (B) Immunostaining for claudin-15 and occludin in colon of WT and Tg mice.

.

The claudin family comprises 27 known members, which form homo and hetero-dimers (Niessen, 2007). 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 5E, 5F and 6B).

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 (Figure 7 and Figure 12, $p<0.05$).

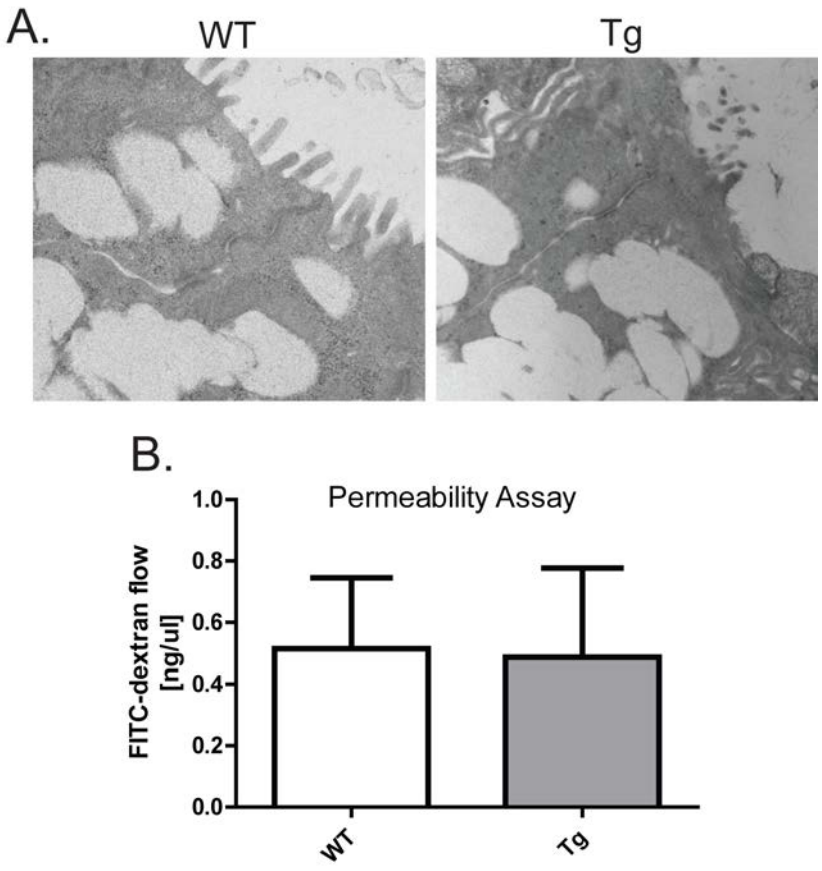


Figure 7. Claudin-1 overexpression does not alter tight junction function in mice

(A) Representative photomicrographs of Electron microscopy images of WT and Tg colons. (B) FITC-dextran flow as assessed via FITC-dextran permeability assay in WT and Tg mice.

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 5D). To evaluate further, we performed Periodic Acidic Schiff (PAS) staining for mucins produced by goblet cells in small intestine (SI) and colon (Figure 8A,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 (Figure 9A, $p<0.001$). To further confirm, Alcian blue staining was used to identify acidic proteins commonly found in mucus-containing cells (Figure 9B). Among the mucins that constitute the colonic mucus barrier, Muc-2 is the most abundant(Coant et al., 2010; Kufe, 2009) 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 8B and 9A).

The secretory goblet cells are one of the four cell types within the intestinal epithelium (van der Flier and Clevers, 2009). 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 8B). 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 8A,B). Staining for lysozyme (marker for Paneth cells; small intestine) did not detect major changes in Cl-1Tg mice compared to WT mice (Figure 8A). 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 8C, $p<0.001$). 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 8D).

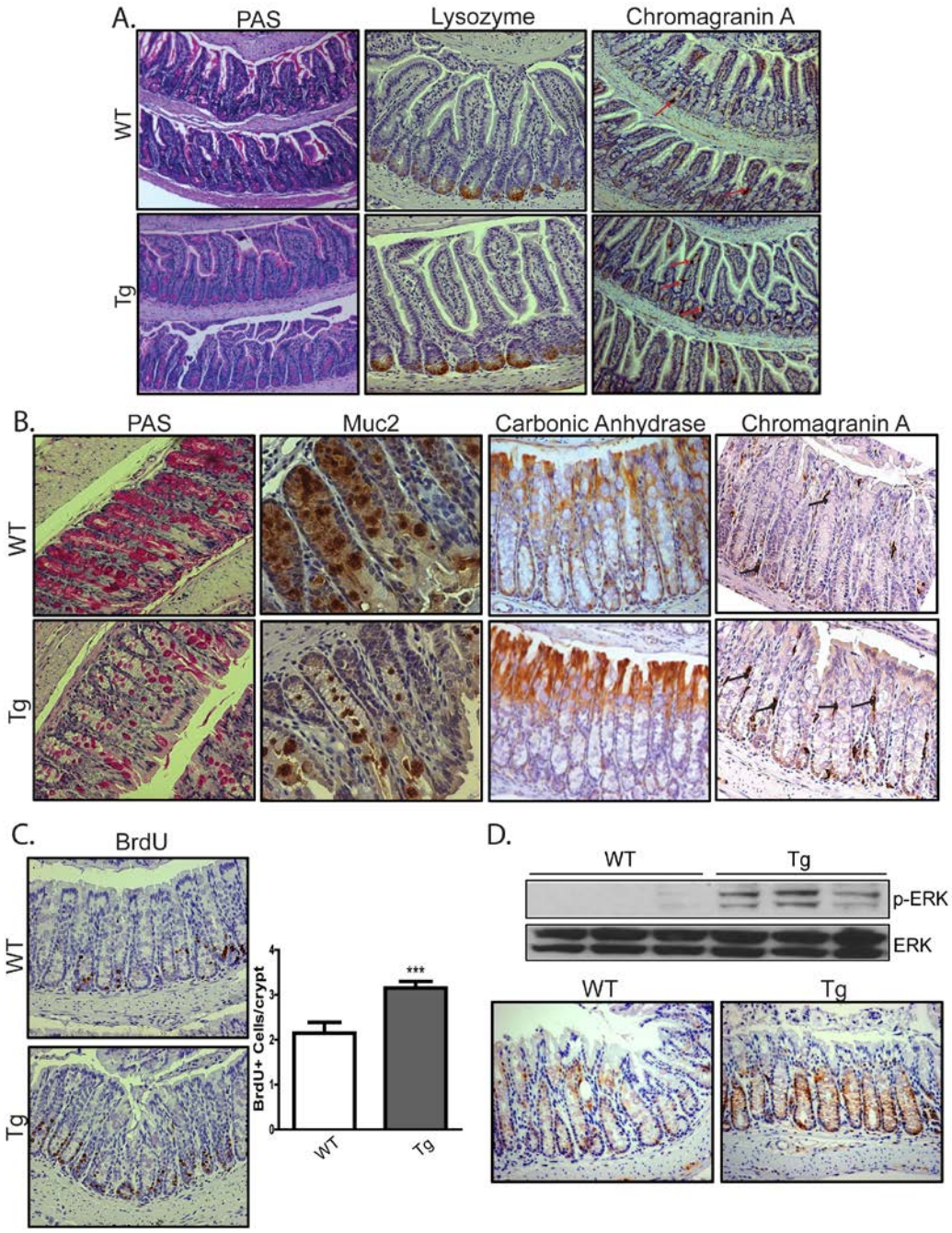


Figure 8. Claudin-1 overexpression alters intestinal epithelial cell lineage and increases proliferation.

(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

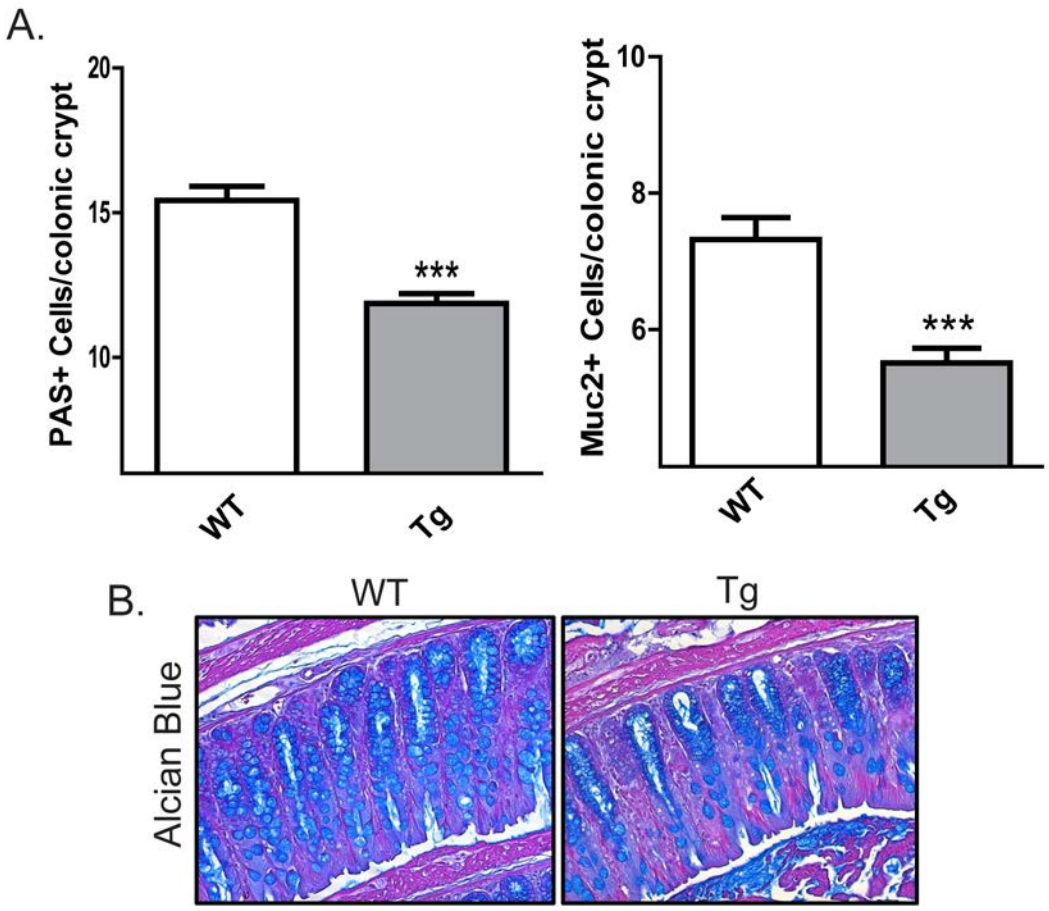


Figure 9. Decreased goblet cell number upon claudin-1 overexpression

(A) Quantification of goblet cells in colon as measured by PAS and Muc2 staining/crypt. (B) Further confirmation showed Claudin-1 overexpression induced a reduction in Alcian blue staining. ***p<0.001

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

Loss of goblet cells characterizes IBD patient samples(Tytgat et al., 1996) and mice with genetic deletion of muc-2 develop spontaneous colitis (van der Sluis et al., 2006). Claudin-1 expression is upregulated in areas of active inflammation in IBD patients (Weber et al., 2008). 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 10A). 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 10B). Histopathological analysis further supported the severity of inflammation in DSS-treated Cl-1Tg *versus* WT mice (Figure 11). However these mice did not recover, lost more than 20% of body weight by day 9 and were therefore euthanized.

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 10C). 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 10C). Furthermore, the recovering Cl-1Tg mice exhibited hyperplastic elongated crypts compared to the extensive normal regenerative crypts in WT mice (Figure 10D). 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 10E). Furthermore, during recovery, Cl-1Tg mice continued to display high scores for both inflammation and epithelial injury while the WT mice recovered almost completely.

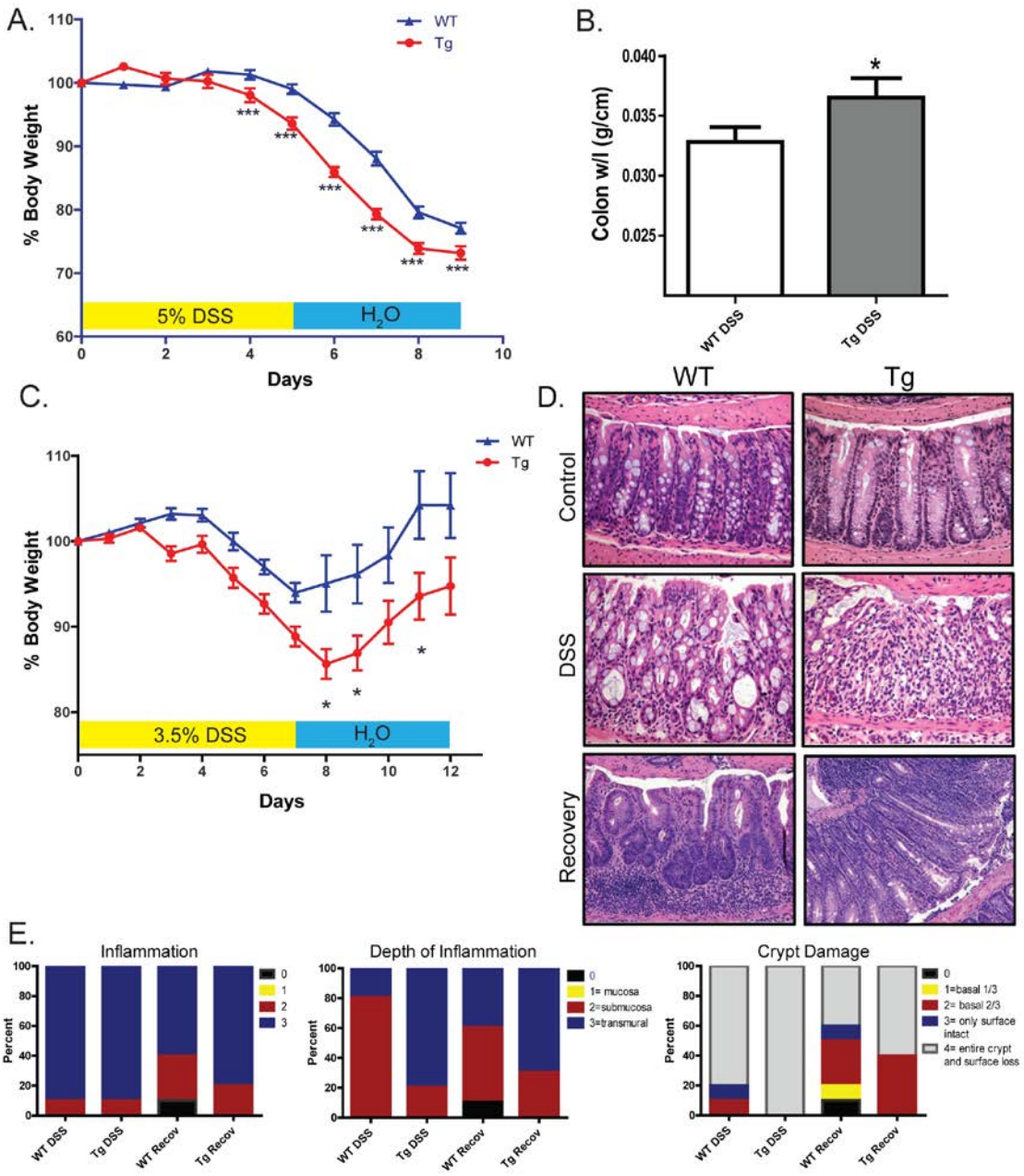


Figure 10. Claudin-1 Tg mice are susceptible to DSS-colitis

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

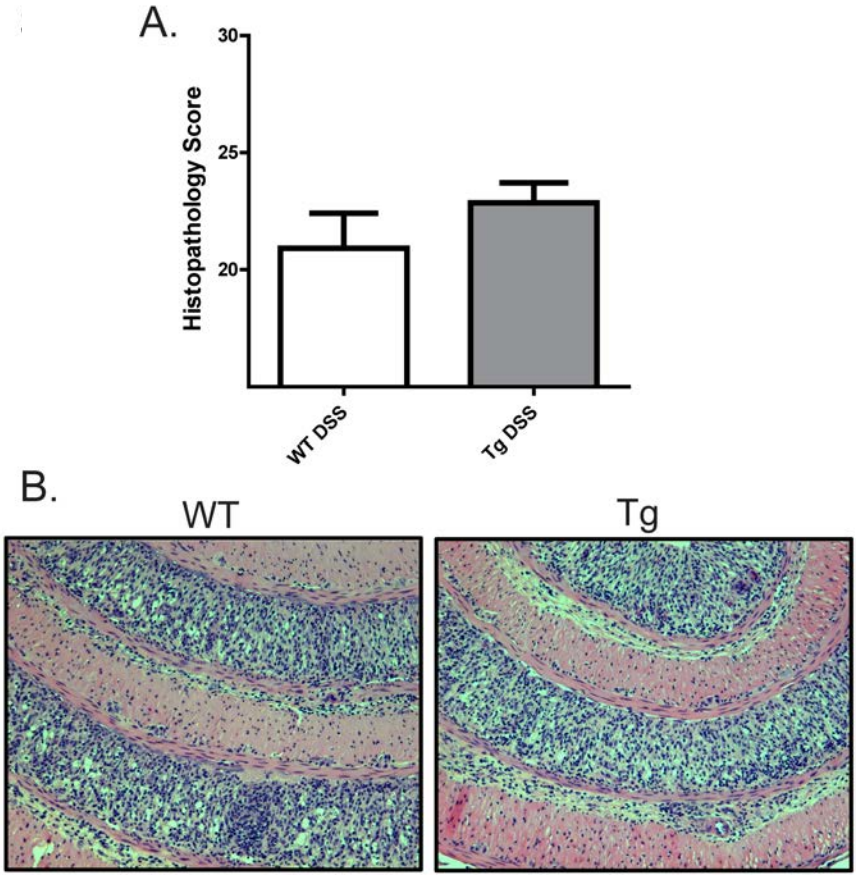


Figure 11. 5% DSS induces severe colitis in WT and Tg mice

(A)Histopathology score of WT and Tg mice treated with 5% DSS. (B) Representative H&E staining of WT and Tg mice treated with DSS.

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 (Kufe, 2009). 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 (Figure 12). 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 13A). 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 (Figure 12). 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 (Figure 12).

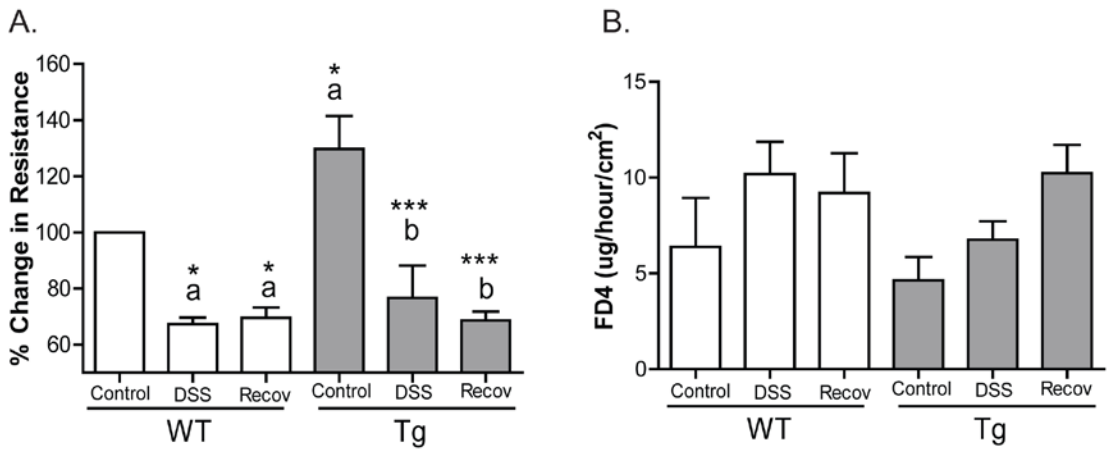


Figure 12. Effect of DSS on colonic transepithelial resistance and FITC (FD4) flux

WT and Tg mice were given DSS and sacrificed on day 4 (DSS) and day 10 (Recovery) and the resistance (A) and FD-4 flux (B) across colonic sheets were measured. [a]compared to WT-control, [b] compared to Cl-1Tg-control, *p<0.05, ***p<0.001

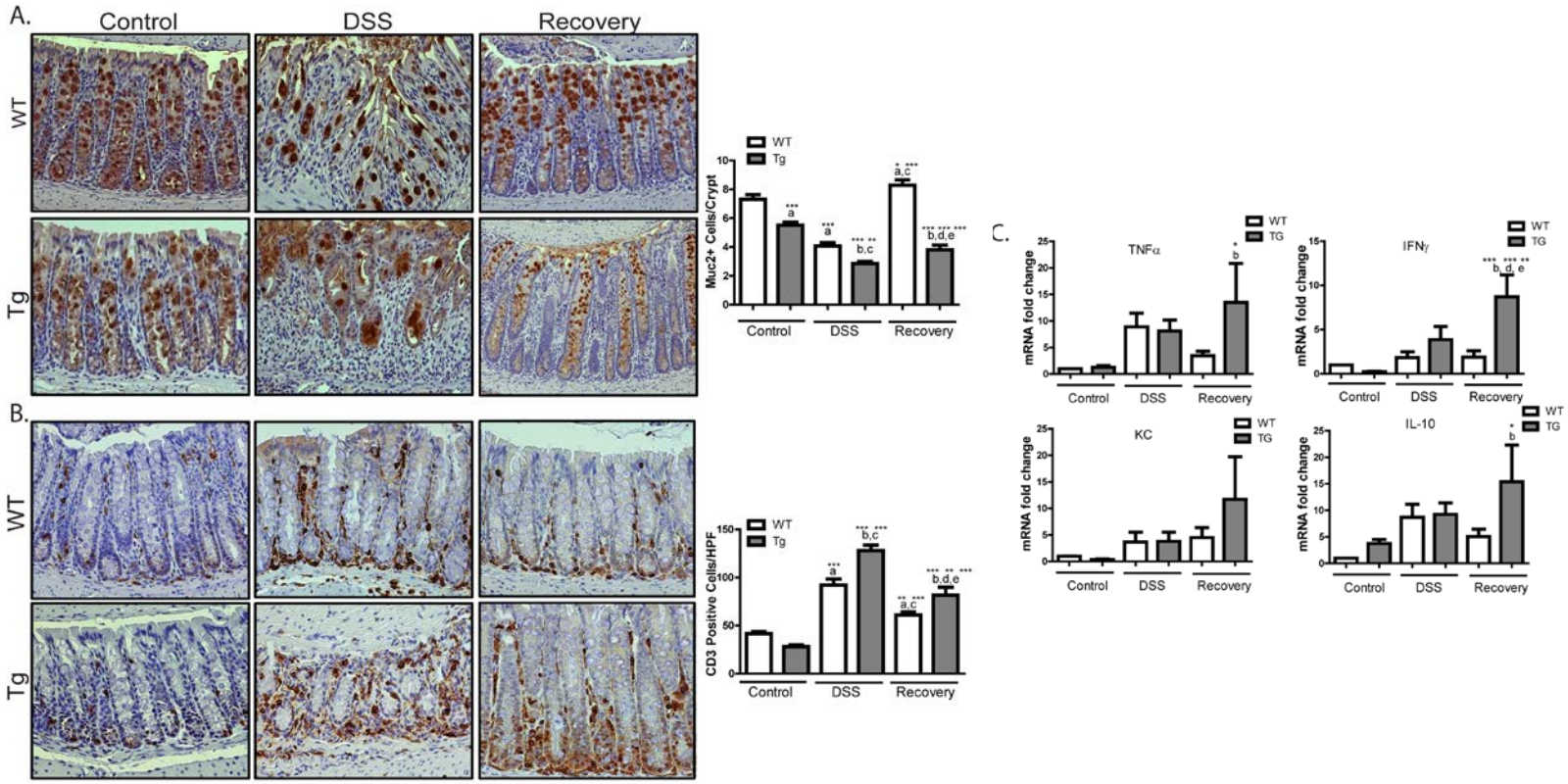


Figure 13. Cl-1 Tg mice have sustained inflammation during recovery from DSS-colitis

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, KC and IL-10 expression. *p<0.05, **p<0.01, and ***p<0.001.

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 ($p<0.001$) (Figure 13A). 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 (van der Sluis et al., 2006).

During colitis there is an infiltration of immune cells accompanied by changes in cytokine gene expression that occurs in response to the ensuing damage (Műzes et al., 2012). 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 13B), 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 TNFα 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

13C). To further confirm these findings, we examined cytokine protein levels using total colon lysates from mice that underwent recovery following DSS-treatment (Figure 14). 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.

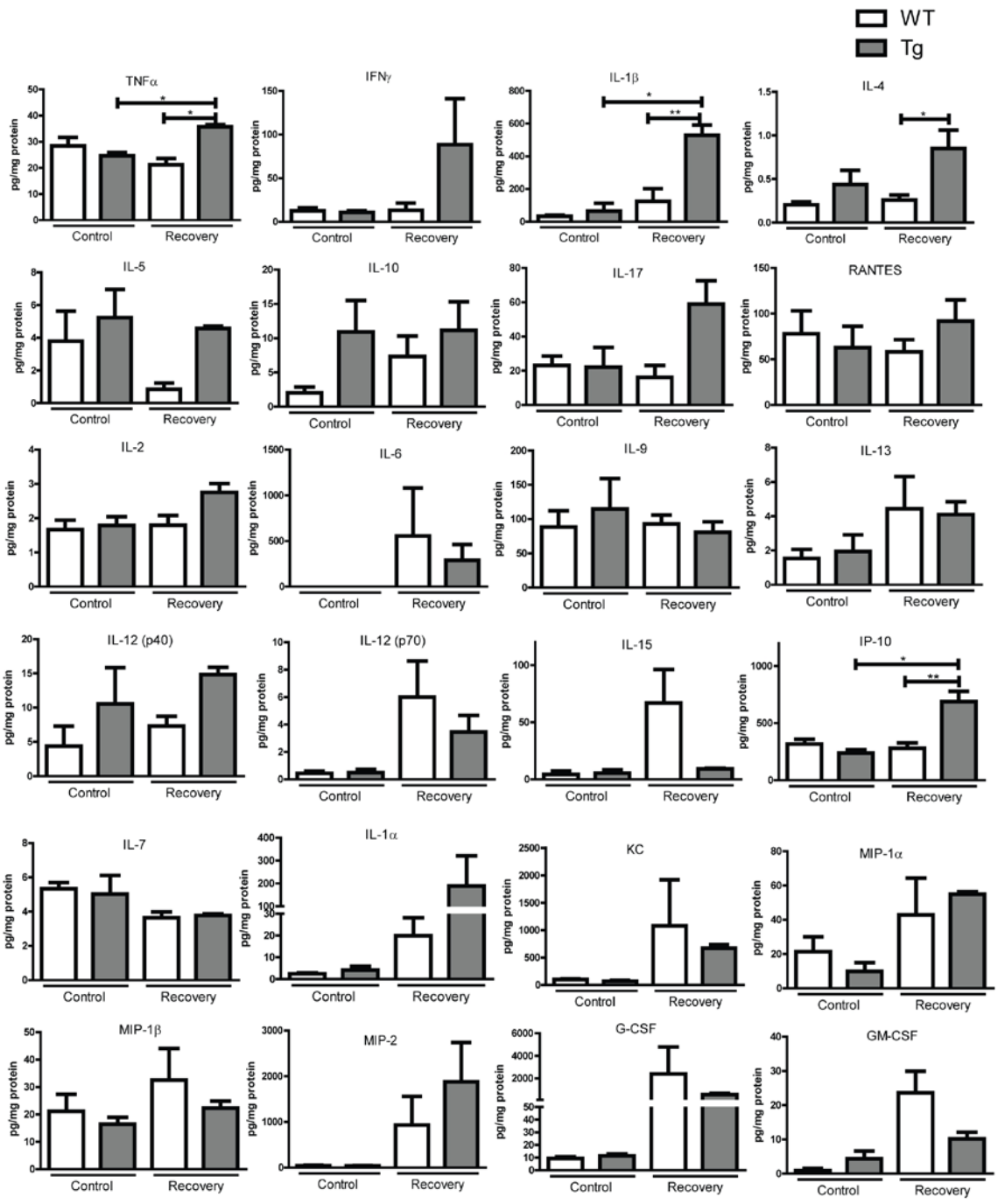


Figure 14. Multiplex Analysis of WT and Tg control and DSS-Recovery mice

WT and Tg colons were harvested after DSS-Recovery and protein isolates were subjected to multiplex analysis. *p<0.05, **p<0.01

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 10D). 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 (Araki et al., 2006). 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 15A,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 15B&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.

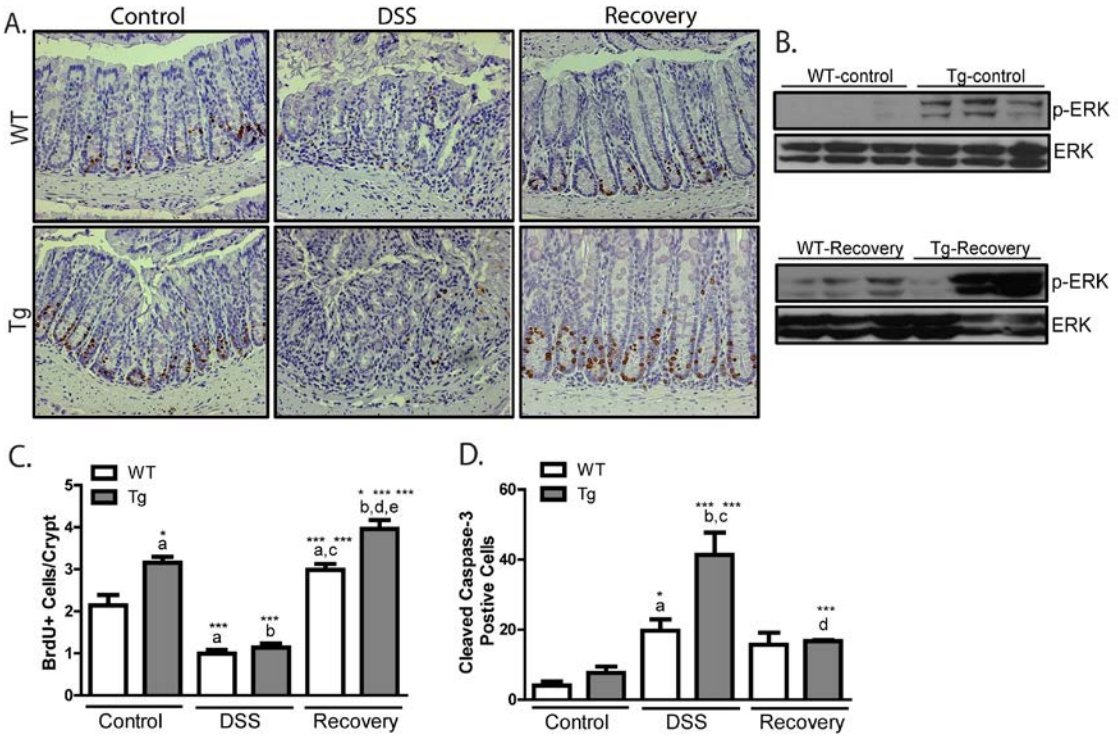


Figure 15. Proliferation and apoptosis are altered in Cl-1Tg mice undergoing DSS treatment and recovery

(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.

Notch-signaling is upregulated in Cl-1Tg mice

The Notch-signaling pathway is the critical regulator of the intestinal epithelial cell fate determination (Fre et al., 2011; Nakamura et al., 2007). Apart, Notch-signaling regulates Muc-2 expression (Okamoto et al., 2009),(van der Flier and Clevers, 2009), and has a critical role in the regulation of mucosal inflammation and proliferation (Okamoto, 2011). 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 16A). 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 (Zheng et al., 2011). Hes-1, inhibits expression of Math1 and thus muc-2, both of which are markers of secretory cell lineage (Qiao and Wong, 2009). 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 16B).

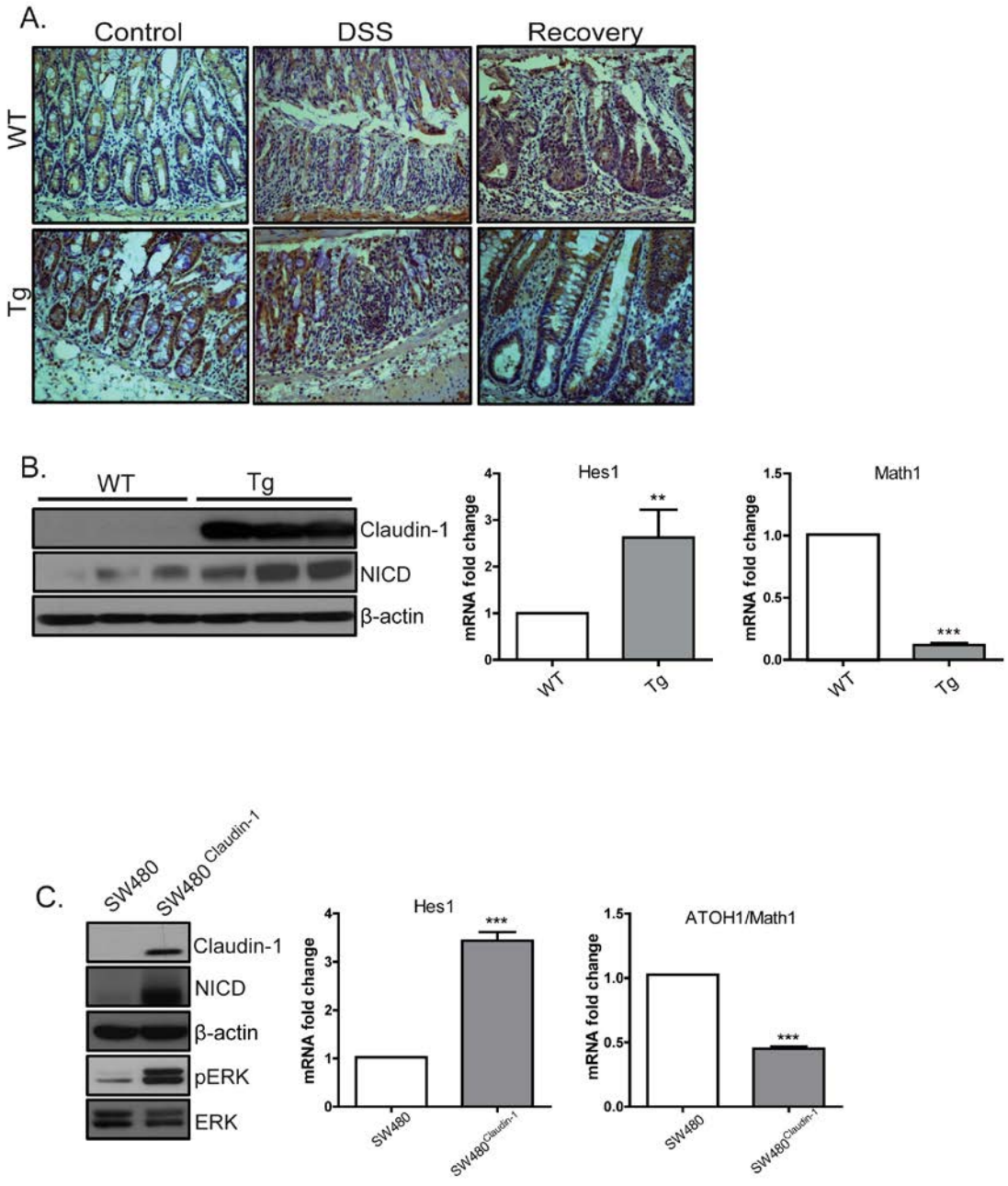


Figure 16. Increased Notch-signaling in Cl-1Tg mice and colon cancer cells during DSS injury and recovery

(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.

We also observed increase in NICD and Hes-1 and a decrease in Math-1 expression in SW480$^{claudin-1}$ cells (stably overexpressing claudin-1) (Figure 16C). 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 17A). 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 17B). Inhibition of Notch-signaling using DAPT reverted the claudin-1-dependent effects upon differentiation and inhibited proliferation in these cells (Figure 17C-E).

An association of claudin-1 with matrix-metalloproteases and potential role in MMP-9 activation is reported (Miyamori et al., 2001). 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 18A). Increased expression of active-MMP-9 was also observed in SW480$^{claudin-1}$ and LS174T$^{claudin-1}$ cells (Dhawan et al., 2005). Similar to Cl-1Tg mice, we also observed increased p-ERK1/2 expression in claudin-1 overexpressing cells (Figure 16C&18C).

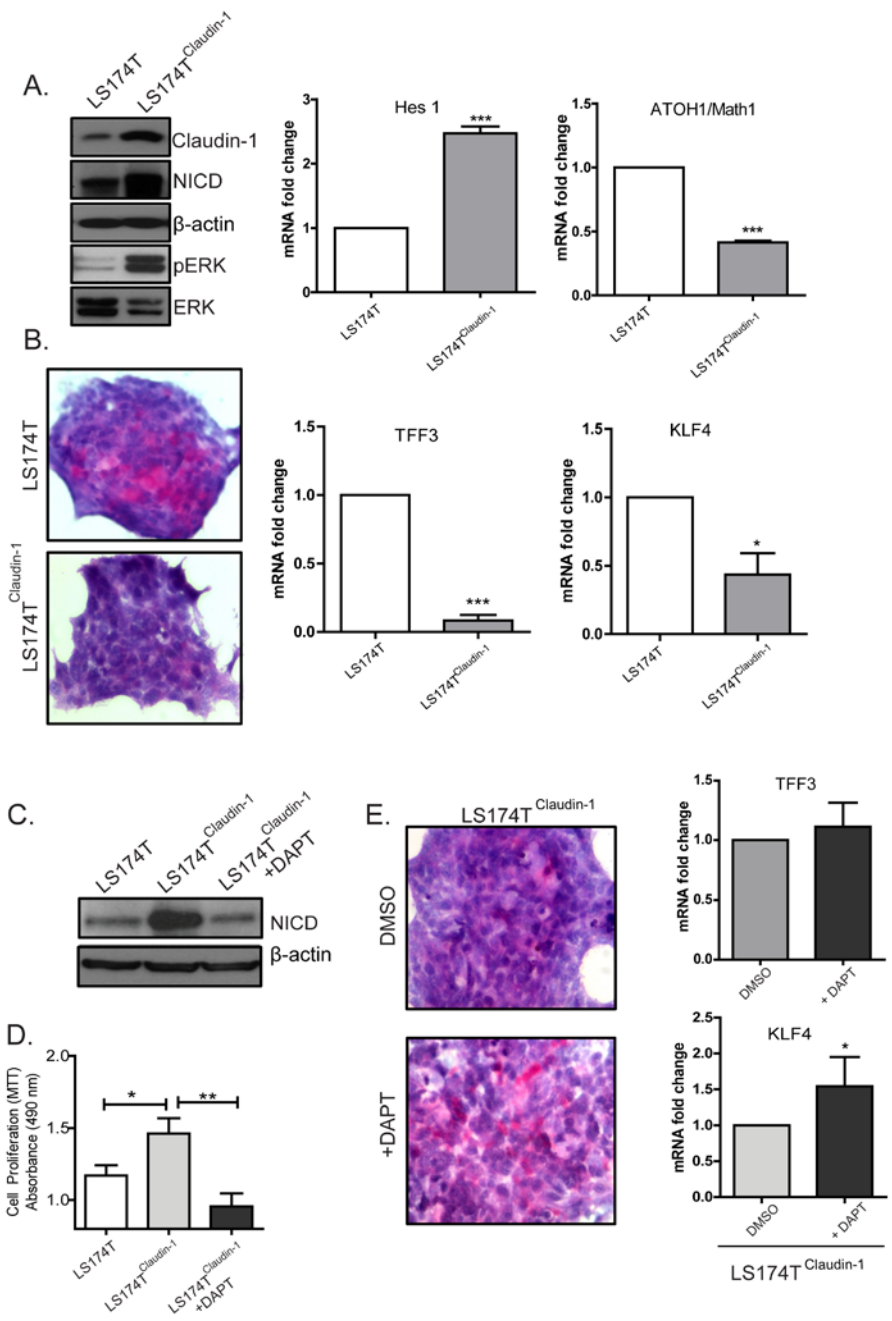


Figure 17. Claudin-1 overexpression alters Notch signaling, proliferation and differentiation in colon cancer cells.

(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

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 18B&C,E,F) without affecting the proliferation and p-ERK1/2 expression (Figure 18C&D). Of note, goblet cell number increases in MMP-9 knockdown mice (Garg et al., 2007). 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 19A-C).

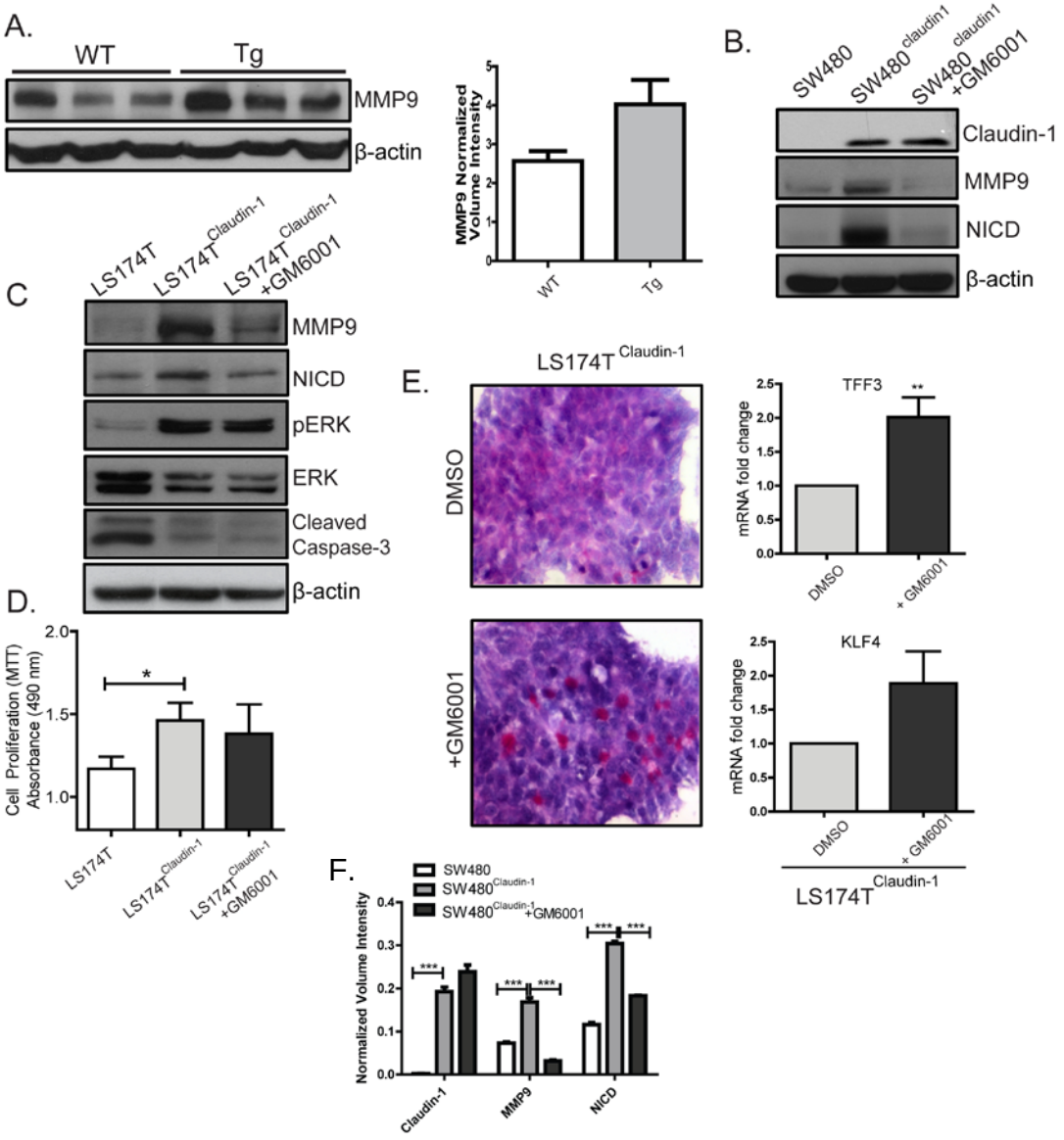


Figure 18. Claudin-1 induced Notch signaling is mediated through the regulation of MMP-9.

(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. (F) Densitometric analysis of GM6001 treatment of SW480$^{Claudin-1}$ normalized to β-actin.

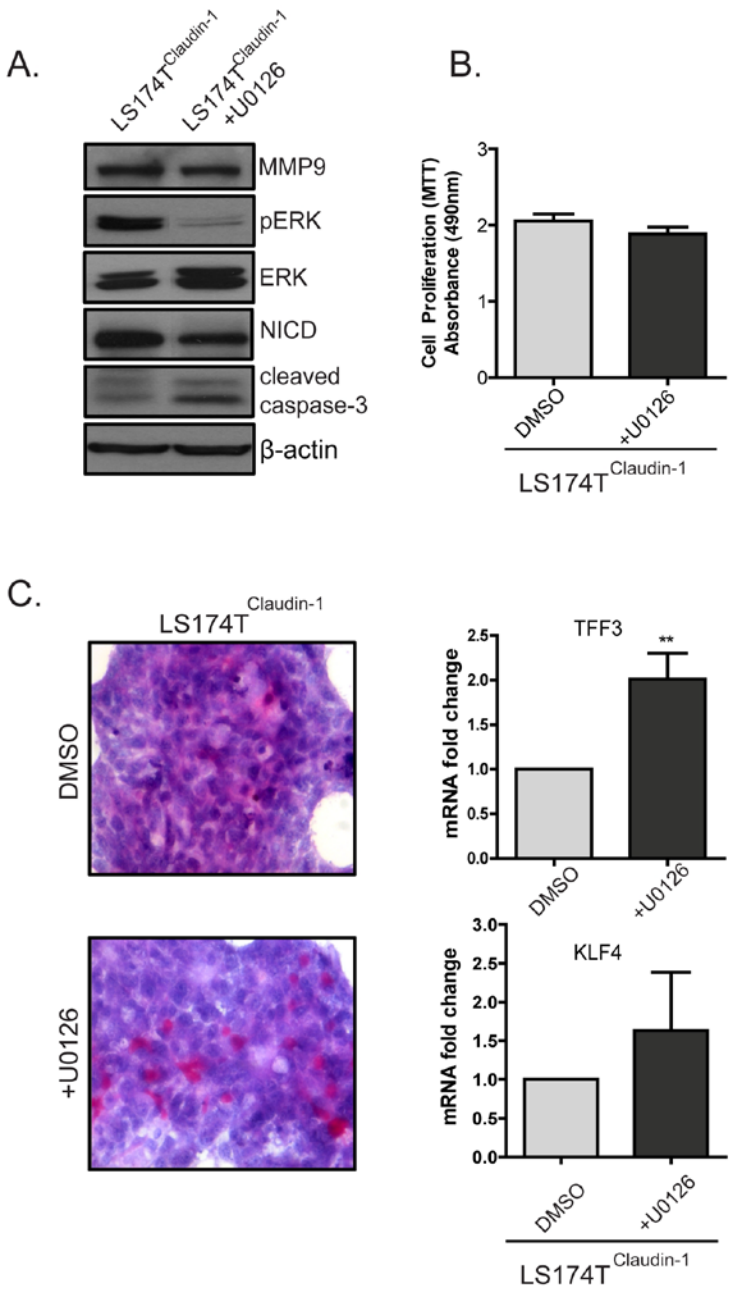


Figure 19. ERK signaling regulates Notch activation, apoptosis and differentiation in Claudin-1 overexpressing cells.

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

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 (Singh et al., 2012; 2011). Recent studies have demonstrated marked increase in claudin-1 expression in colon cancer (Dhawan et al., 2005) as well as the areas of active inflammation and its correlation with neoplastic transformation (Kinugasa et al., 2010; Weber et al., 2008). However, no study to 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 (van der Flier and Clevers, 2009; VanDussen et al., 2012). The principal secretory product of goblet cells is muc-2, a key constituent of the mucus layer that protects the mucosal epithelial layer (Bergstrom et al., 2010; Johansson et al., 2008). Notably, Notch activation and muc-2/goblet cell depletion is a characteristic associated with mucosal inflammation and colon cancer (Garg et al., 2011; Maloy and Powrie, 2011; Qiao and Wong, 2009; van der Sluis et al., 2006; K. Yang et al., 2008; W. Yang et al., 2005). 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 (Vooijs et al., 2011) 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 regulates 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 (Dhawan et al., 2005; Oku et al., 2006). 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 (Sawey et al., 2007). Furthermore, Notch-activity is modulated in the colon of mice genetically manipulated for MMP-9 expression/activity (Garg et al., 2007). 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 baseline increases 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-1Tg 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 (Garg et al., 2007; van der Sluis et al., 2006).

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 (Fang et al., 2012). 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,(Yeruva et al., 2008) and IFN-γ is the major inducer of IP-10 (Cassatella et al., 1997). Furthermore, IL-1β has also been recently shown to induce IP-10, especially in combination with TNFα and IFN-γ (Yeruva et al., 2008). Notably, in recovering Cl-1Tg mice there was sustained increase in TNFα, 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 (Figure 9 & 12). 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 (Evans et al., 2007). Also, claudin-1 is also a co-receptor for Hepatitis-C-virus (Evans et al., 2007). 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 the

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 (Suh et al., 2012). Co-operation between Notch and ERK1/2-signaling in the regulation of proliferation and differentiation has been reported (Sundaram, 2005). Ras-activated breast cancer cells acquire tumorigenic properties when Notch signaling is activated (Mittal et al., 2009). 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 (Dhawan et al., 2005). Taken together, our data support a novel role of claudin-1 in the regulation of Notch-signaling and colonic homeostasis.

CHAPTER III

INTESTINAL EPITHELIAL CELL SPECIFIC CLAUDIN-1 EXPRESSION ENHANCES SUSCEPTIBILITY to APC-MEDIATED COLON TUMORIGENESIS

Introduction

Claudin-1 is a member of the claudin family of tight junction family of proteins whose traditional roles involve maintenance of the epithelial barrier. In recent years many claudins have been shown to be important players in several types of cancers, in capacities beyond barrier regulation, when their expression patterns have been altered. In most of these studies, loss of TJ proteins have contributed to deregulation of mechanical aspects of tumor progression such as migration (Ikari et al., 2011), and invasion (Oku et al., 2006; Zavala-Zendejas and Torres-Martinez, 2011). We and others have shown that the claudin-1 expression is upregulated in human colon cancer (Dhawan et al., 2005) and that modulation of this expression regulates the primary tumor growth and metastasis in xenograft models. It is not quite clear, however, how increased expression of claudin-1 contributes to colon tumorigenesis.

Importantly, in our recent studies, we have demonstrated a role for claudin-1 in the maintenance of normal intestinal homeostasis whereby intestinal epithelial cell (IEC)-specific constitutive expression of claudin-1 altered goblet cell differentiation by promoting Notch activation (Pope et al., 2013). Importantly,

Villin-claudin-1 Tg mice also demonstrated enhanced severity of DSS-induced colitis and impaired recovery from colitis-induced epithelial injury, which was attributed to the decreased mucosal protection due to loss of the primary component of goblet cells and defense, Mucin-2. This further confirmed a previously established connection between inflammation and claudin-1 expression that has also been shown in studies of active IBD as well as colitis-associated cancer (Weber et al., 2008), (Kinugasa et al., 2010).

Sole overexpression of claudin-1, however, was not sufficient to induce tumorigenesis in mice. Therefore, to better determine the role of claudin-1 in colon tumorigenesis, we crossed the Villin-claudin-1 Tg mouse with the APC^{Min} (APC) mouse model of intestinal tumorigenesis. The Min model replicates the common mutation inherited in Familial Adenomatous polyposis disease (FAP) which predisposes patients to spontaneous colorectal cancer. Although APC mice have been shown to develop adenomas of the small intestine, they rarely develop those of large bowel origin (McCart et al., 2008). Yet much of the work performed in these mice has helped make strides in the understanding of colon cancer. Consequently, this model has been widely used to study the role of specific genes of interest in colorectal cancer in conjunction with the APC genetic mutation, and in some cases have been able to drive colon tumorigenesis. Indeed, some instances where colon tumors were initiated in other models of APC mediated tumorigenesis involved direct or indirect activation of Notch signaling (Fre et al., 2009), (Peignon et al., 2011).

Here, we show that claudin-1 overexpression in APC mice causes increased tumor development in the colon and decreases survival. Additionally tumors in APC-Cldn1 mice have elevated Wnt signaling and Notch activity due to claudin-1 upregulation. Lastly, increased colon tumorigenesis in these mice demonstrates upregulated pro-inflammatory signature including IL-23/IL-17 immune signaling. Notably, increased mucosal permeability and bacterial translocation, as seen in APC-Cldn1 mice, upregulates IL-23/IL-17 signaling to promote colon cancer. Taken together, our current studies provide a clear insight into the role of claudin-1 protein in the regulation of colonic homeostasis and colorectal cancer.

Materials and Methods

Mice

To obtain $APC^{Min/+}$-Villin-Cldn1 Tg mice (APC-Cldn1), APC^{Min} (APC) males, purchased from Jackson Laboratories (Bar Harbor, Maine) were bred with Villin-Claudin-1-Tg females. Claudin-1 overexpression was assessed by PCR as described previously (Pope et al., 2013) on genomic DNA isolated from tail snips using DNA isolation buffer (Viagen Biotech). Identification of mutated APC allele was performed using a modified protocol from Jackson Laboratories and a set of three primers identifying the wild type and mutant alleles. APC and APC-Cldn1 littermates were monitored for signs of morbidity including hunched posture, anemia, and body weight loss and sacrificed according to the guidelines of

Vanderbilt University Institutional Animal Care and Use Committee (IACUC). Accelerated tumorigenesis was induced by administering 8-10 week old mice dextran sulphate sodium in their drinking water. Mice were monitored throughout duration of the experiment as described previously (Pope et al., 2013), and examined intermittently via colon endoscopy.

Tissue Processing

As described previously, the colon and small intestine were dissected and flushed with PBS, opened flat and formalin fixed using the Swiss roll method. Further processing was performed by the Vanderbilt Translational Pathology Shared Resource Core. Distal and proximal sections of the colon were snap-frozen and stored at -80°C for further analysis. Where applicable, colonic tumors were quantified. Tumors were either isolated and frozen for further analysis, or left in the colon to be processed for embedding and H&E staining.

Tumor size measurements

Tumor area was calculated using Axiovision digital imaging processing software by outlining tumors of three to four mice each of APC and APC-Cldn1 mice.

Immunohistochemistry

Immunostaining of paraffin-embedded tissues was performed as described previously (Pope et al., 2013) using VectaStain ABC kit (Vector Laboratories) and the indicated antibodies. Images were obtained using a Zeiss light microscope.

RNA isolation

Total RNA was isolated from tumors excised from the colon of APC and APC-Claudin-1 mice using Qiagen RNAeasy Mini kit with DNAse digestion step performed. The integrity of the RNA was determined by performing formaldehyde gel electrophoresis. Samples displaying two bands, corresponding to the 18S and 28S subunits, and having an A260/A280 of ~1.8 were used for experiments.

Microarray Analysis

Total RNA was isolated from snap-frozen tumors, as described above, and RNA integrity was measured. Samples were submitted to the Vanderbilt Microarray Shared Resource for DNA affymetrix array.

Quantitative reverse transcription-PCR

Total RNA (1ug) of each sample was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Each qRT-PCR reaction contained SYBR Green Master Mix, the indicated primer sets and 25ng of cDNA. Samples were loaded in triplicates on 96 well plates and run on a Bio-Rad iCycler. Ct values were utilized to calculate fold change and normalization was performed using beta-actin.

Permeability Assay

Assessment of intestinal permeability to 4kDa FITC-dextran was performed as described previously (Pope et al., 2013).

Bacterial Translocation

Translocation of bacteria was assessed by detecting the amount of bacterial 16S rDNA using qRT-PCR and specific primers for conserved regions of the bacterial 16S rDNA (Fu et al., 2011). Genomic DNA was isolated from the distal colon of three mice per group and 25ng was used per reaction. The mouse Selp gene was utilized as normalization gene.

Statistics

Statistical analyses were performed using Graphpad Prism software (San Diego, CA) for t-test analysis where comparisons between two groups were involved. SPSS software (College Station, TX) was utilized for analyses of Logistic regression (for binary outcomes) and Chi2 (for categorical). *P* values less than 0.05 were considered significant.

Results

Claudin-1 overexpression increases colon tumorigenesis and decreases survival

Increased claudin-1 expression has been frequently observed in colon cancer, however the consequences of the in-vivo upregulation in colonic epithelial cells have not been investigated. To determine the role of claudin-1 in colon tumorigenesis, we crossed Villin-Claudin-1-Tg mice with APCMin mice (APC) to generate APCMin-Villin-Claudin-1 (APC-Cldn1) mice. APC mice

characteristically develop adenomas in the small intestine with little to no tumor occurrence in the colon (McCart et al., 2008). Among mice that developed colonic tumors, we observed that APC-Cldn1 (n=18) mice developed colonic tumors at a higher frequency (p=0.0003) than APC mice (n=18) (Figure 20A). Endoscopy of mouse colon at 10 weeks of age showed that APC-Cldn1 mice develop colonic tumor before APC mice, suggesting a decrease in tumor latency (Figure 21). Interestingly, the few colonic tumors that developed in APC mice were localized towards the middle of the colon whereas the tumors in APC-Cldn1 mice were more distally localized, as shown in Figure 20B. Upon examination of the H&Es, it also appeared that the tumors were larger in APC-Cldn1 mice; therefore we measured the size of tumors using imaging analysis software, and indeed found a significant increase (p=0.0178) in the tumor area of APC-Cldn1 mice compared to that of APC (Figure 20C,D). Histological analysis further showed that the APC-Claudin-1 tumors were less differentiated than APC (Figure 20D). Additionally, APC adenomas were predominately low grade, while the majority of APC-Cldn1 tumors have high-grade dysplasia (p=0.0007) (Table 3). It is rare that adenomas of APC mice, originating from the colon or small bowel progress to invasive adenocarcinoma, yet we were able to detect an incident of invasion in the APC-Cldn-1 mice (Figure 20E, Table 3). Through routine care and observation of the mice, we also noticed that APC-cldn1 mice began showing signs of morbidity much sooner than APC mice. The average life span of a Min mouse is approximately six months. To determine if there was a significant difference in survival, we plotted a Kaplan Meir curve and found that

APC-cldn1 mice (n=40) have a statistically significant less survival (p=0.0027) than APC mice (n=43) (Figure 20F). As mentioned previously, multiple adenoma formation is restricted to the small intestine in the APC model and is thought to attribute to their limited life span. Therefore we thought it important to assess whether changes were observed between tumors of the small intestine also progress with claudin-1 overexpression. However, we observed no significant difference in the intestinal tumor number with claudin-1 overexpression (Figure 22) and although more APC-Cldn1 mice presented tumors with high-grade dysplasia, this difference was not significant (Table 4). Taken together, these results suggest claudin-1 overexpression enhances susceptibility to tumor development in the colon of APC mice as well as contributes to tumor progression.

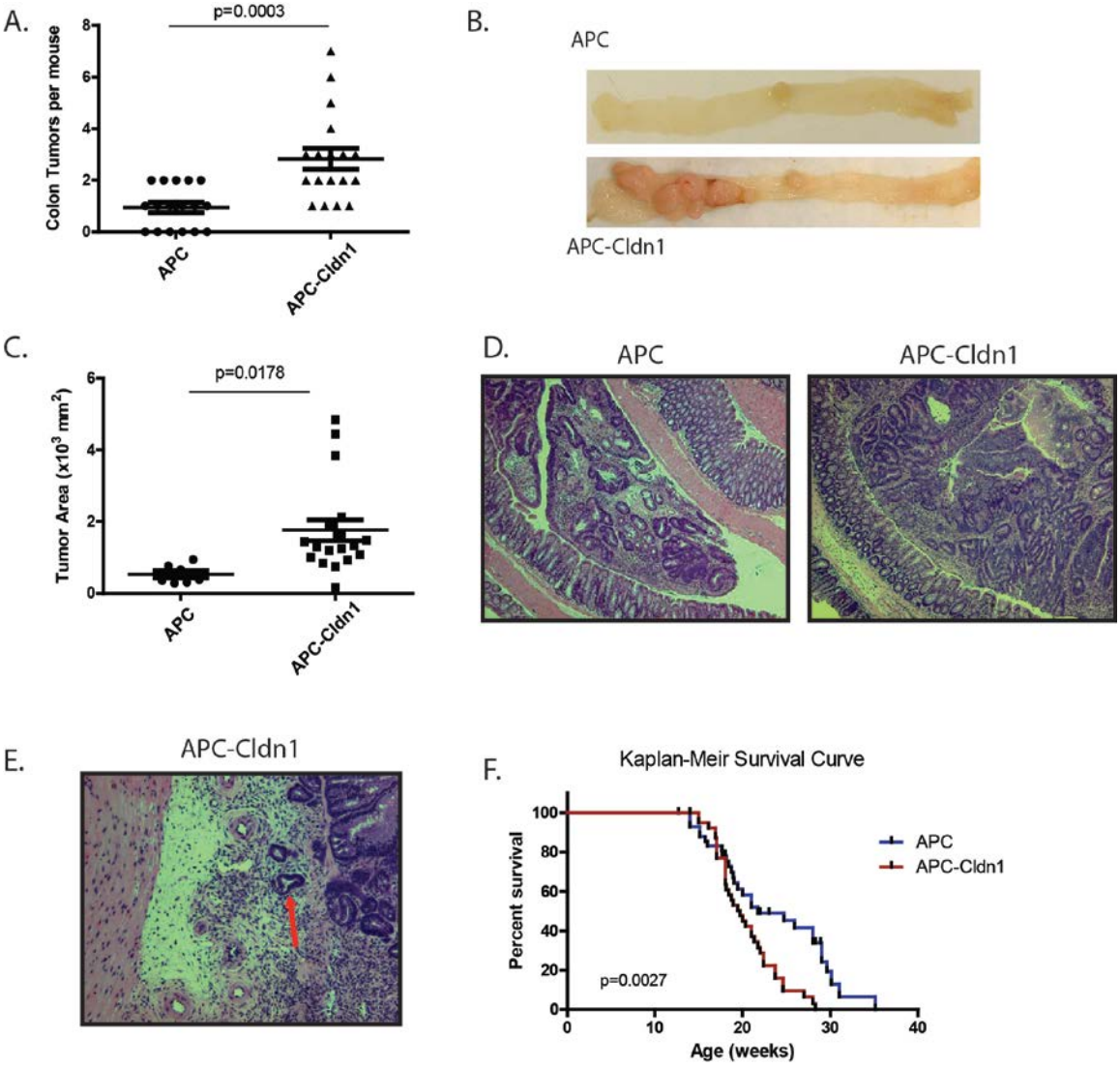


Figure 20. Claudin-1 overexpression increases colon tumorigenesis in APC mice and decreases survival.

(A) Increased number of colon tumors in APC-Cldn1 mice. (n=18 APC mice, n=17 APC-Cldn1 mice). (B) Representative images of sporadic colon tumors. (C) Increased tumor size in APC-Cldn1 tumors. (D) Representative H&E stainings. (E) Invading carcinoma in APC-Cldn1 mouse. (F) Kaplan Meir survival curve between APC and APC-Cldn1 mice, p=0.0027.

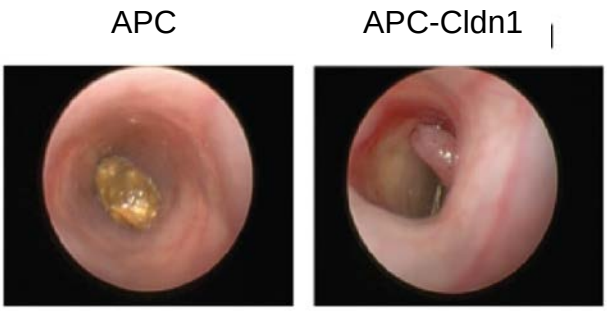


Figure 21. APC-Cldn1 tumor appears before those of APC mice

Colonoscopy of age matched APC and APC-Cldn1 mice at 10 weeks of age.

Table 3. Comparative Histological Analysis Colon Tumors

Genotype	No. mice with tumors	Average no. tumors per mouse*	Total no. mice w/ HGD**	Total no. Invasive adenocarcinomas	Total no. of adenomas
APC (*n=18*)	15	1.28	2	0	22
APC-Cldn1 (*n=16*)	16	2.76	11	1	45

Abbreviation: HGD, high grade dysplasia.
**p=0.003*
***Logistic regression for likelihood of developing HGD p=0.0007*

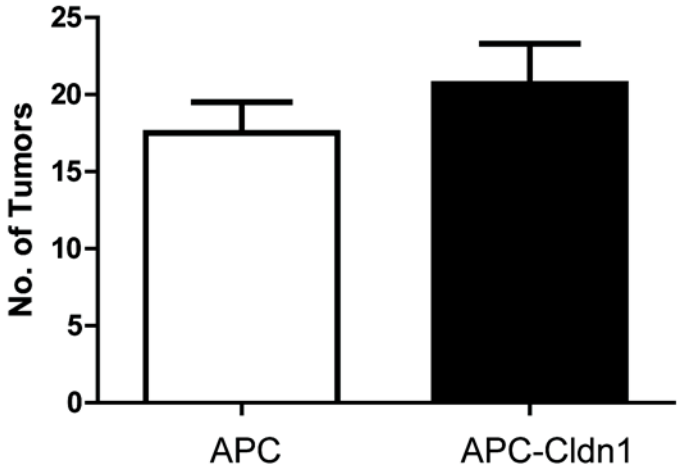


Figure 22. Quantification of Small Intestine Adenomas

Adenomas of small intestine from APC and APC-Cldn1 mice were quantified.

Table 4. Comparative Histological Analysis of Small Intestine Adenomas

Genotype	Average no. tumors per mouse	Total no. w/ HGD
Small Intestine		
APC *(n=14)*	17.5	4
APC-Cldn1 (*n=17)*	20.5	7

APC-Claudin-1 tumors have increased Wnt/Notch signaling

An increase in tumor size or number usually results from an increase in proliferation and/or an associated decrease in apoptosis. Therefore to assess proliferation, we performed immunostaining for Ki67, a well-known marker of cellular proliferation. We quantified a significant increase ($p=0.0125$) in tumor proliferation of APC-Cldn1 mice compared to APC (Figure 23A). We observed no significant difference in apoptosis as measured by immunostaining for cleaved caspase 3 (Figure 24). As Claudin-1 is a downstream target of Wnt signaling (Miwa et al., 2001), and tumors that arise from the loss of APC have constitutive Wnt activation, we decided to examine the colonic tumors in each of these mice for potential upregulation of the Wnt/β-catenin pathway. We immunostained tumors for β-catenin, the primary effector of Wnt activation, to assess for nuclear/cytoplasmic staining, which is an indication of activated β-catenin. APC-Cldn1 tumors showed a noticeable increase in nuclear and cytoplasmic staining compared to that of APC tumors (Figure 23B). To further assess Wnt upregulation, we performed qRT-PCR analysis using total RNA isolated from the colonic tumors of APC and APC-Cldn1 and detected increased mRNA expression of established Wnt target genes CD44 ($p=0.0159$), Lgr5 (5-fold increase), and Axin2 (3-fold increase) (Figure 23C). Previously, we have shown that intestinal specific overexpression of claudin-1 in mice causes activation of Notch signaling (Pope et al., 2013). Additionally, several studies have shown a role for the Notch pathway in colon tumorigenesis and targeted therapy (Miyamoto et al., 2013), (Sonoshita et al., 2011). Indeed, we were able to

confirm Notch upregulation in colonic tumors by observing an increase in Hes1 and significant decrease ($p<0.0001$) in Math1 mRNA expression (Figure 23D). Taken together, we can conclude that increased claudin-1 expression aids in tumor progression via upregulation of Notch and Wnt signaling pathways.

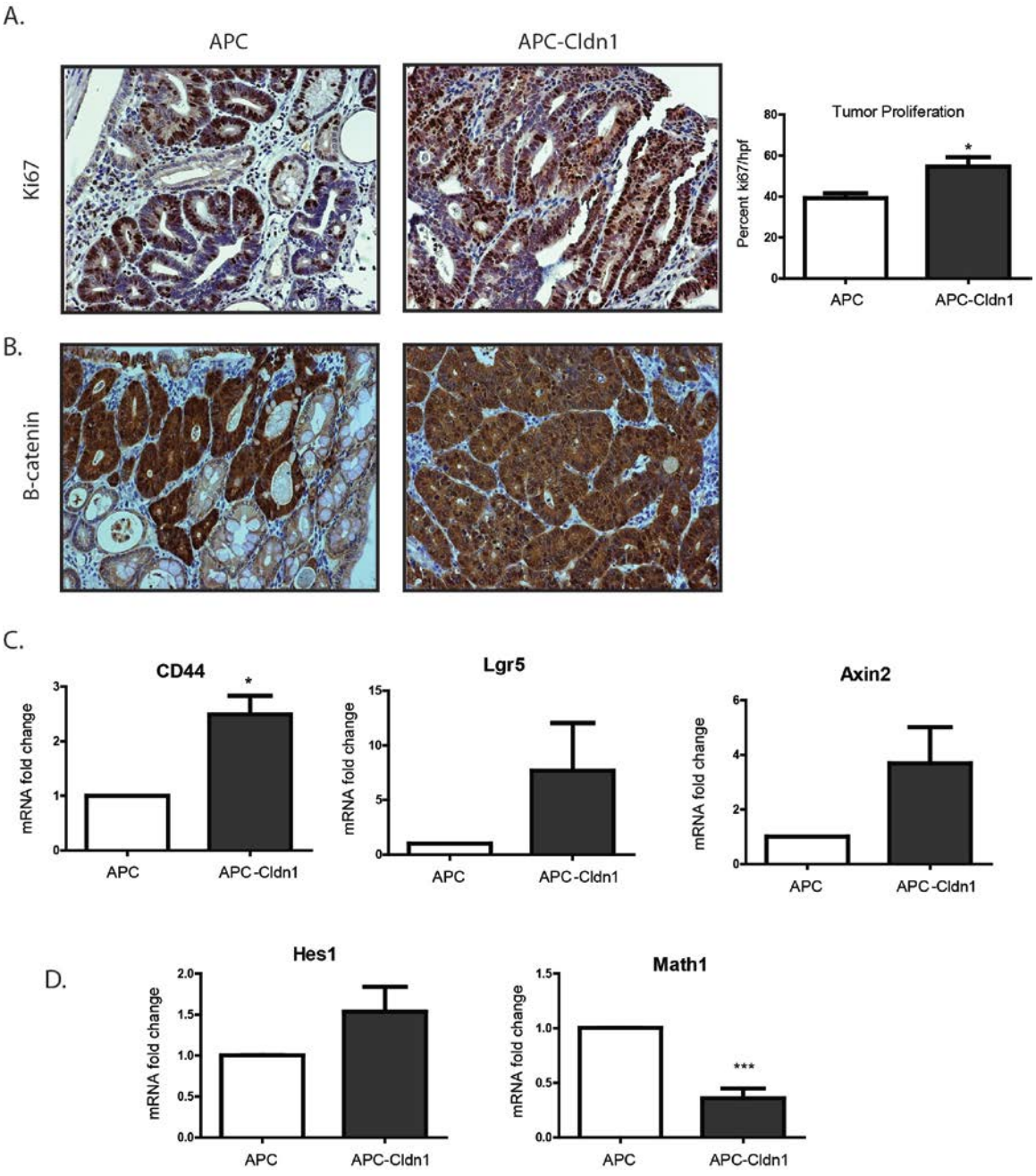


Figure 23. Increased proliferation and upregulated Wnt/Notch Signaling contributes to increased tumorigenesis in APC-Cldn1 mice

(A) Immunostaining and quantification of Ki67 shows increased proliferation in APC-Cldn1 tumors. (B) Nuclear/cytoplasmic β-catenin staining is increased in tumors of APC-Cldn1 mice compared to APC mice. (C) qRT-PCR analysis of Wnt target genes and (D) Notch target genes in tumors of APC and APC-Cldn1 mice. *$p<0.05$, ***$p<0.001$

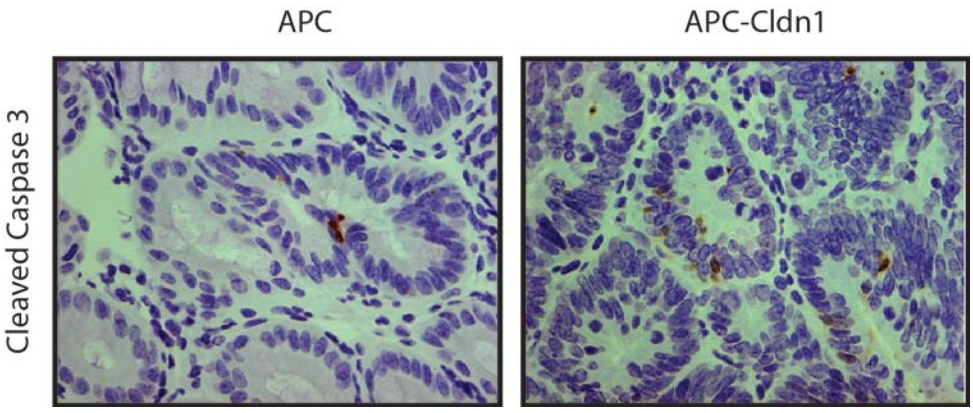


Figure 24. Apoptosis was not significantly altered in tumors from APC-Cldn1 mice

Cleaved caspase-3 staining from adenomas of APC and APC-Cldn1 mice.

APC-Claudin-1 tumors have increased inflammation

To further assess global changes that occur as a result of claudin-1 upregulation we performed microarray analysis of colonic tumors isolated from APC and APC-Cldn1 mice. Figure 25 shows a list of selected genes that were upregulated and downregulated by a factor of at least 1.5 fold or greater in APC-Cld1 mice. In accordance with the data shown above, tumors of APC-Cldn1 mice showed altered expression of genes correlative with increased Wnt and Notch activities.

Additionally, Notch target genes known to regulate mucosal defense were shown to be downregulated in APC-Cldn1 tumors. To validate mRNA expression of these genes, we performed qRT-PCR analysis (Figure 26A). Mucin-2, a primary component of goblet cells which has previously been shown to be

downregulated with claudin-1 overexpression(Pope et al., 2013), was significantly decreased (p=0.0022) in APC-Cldn1 tumors. Kruppel-like factor 4 (Klf4) and Trefoil factor 3 (Tff3), also known regulators of goblet cell development and mucosal defense, were also decreased in APC-Cldn1 tumors (p=0.0031). Studies assessing the loss of Muc2 and Klf4 in the role of APC mediated tumorigenesis have shown an increase in colon tumorigenesis as a result of inflammation (K. Yang et al., 2008) (Ghaleb et al., 2007). Additionally, we observed several genes corresponding to inflammation and the immune response in our microarray analysis. These observations hinted at a possible mechanism for tumor progression involving inflammation. We performed qRT-PCR analysis for common cytokines upregulated during inflammation and observed a significant increase in IL-10 mRNA expression (p=0.0139), with an accompanied increase in IP-10 and TNFα (2- and 3-fold increase, respectively) (Figure 26B). These immune regulators have been shown to be upregulated in response to systemic inflammation, most notably observed in diseases such as colitis.

A.

Wnt Signaling Related Genes		
Gene Symbol	p-value	Fold Change
Tcf4	0.068	1.581
Lef1	0.017	2.238
Fzd10	0.016	2.219
Axin2	0.040	1.693
Wnt6	0.022	1.886
Wnt10a	0.125	1.911
Sox17	0.064	2.694
Ephb6	0.029	2.527

B.

Notch Signaling Related Genes		
Gene Symbol	p-value	Fold Change
Tcf4	0.068	1.581
Lef1	0.017	2.238
Fzd10	0.016	2.220
Axin2	0.040	1.694
Mmp9	0.042	1.628
Atoh1	0.047	-3.487
Klf4	0.010	-2.495
Muc2	0.112	-2.141
Muc3	0.002	-6.700
Muc4	0.345	-1.590
Tff3	0.184	-1.789

C.

Inflammation/Immune Response Genes		
Gene Symbol	p-value	Fold Change
IL23a, p19	0.011	2.250
Cxcl5	0.060	2.392
Cxcl9	0.134	2.394
Cxcl2	0.462	1.548
Mmp9	0.042	1.628
Nfat5	0.031	1.515
Pla2g5	0.002	2.796
Retn1b	0.045	10.865
Lcn2	0.001	1.894
Ccl6	0.075	1.658
Ccl28	0.002	5.352
Ccl3	0.001	1.894
IL1b	0.048	1.825
Csf3r	0.056	1.567
Akt2	0.001	1.541
H2-AA	0.017	1.764
H2-Eb1	0.051	1.666
CD244	0.010	-2.207
Tff3	0.184	-1.790
Muc2	0.112	-2.141

Figure 25. Microarray analysis of APC-Cldn1 vs APC Tumors

Genes altered with claudin-1 overexpression, with at least 1.5 fold change, involved in (A) Wnt Signaling (B) NOTCH signaling or (C) Inflammation/Immune response.

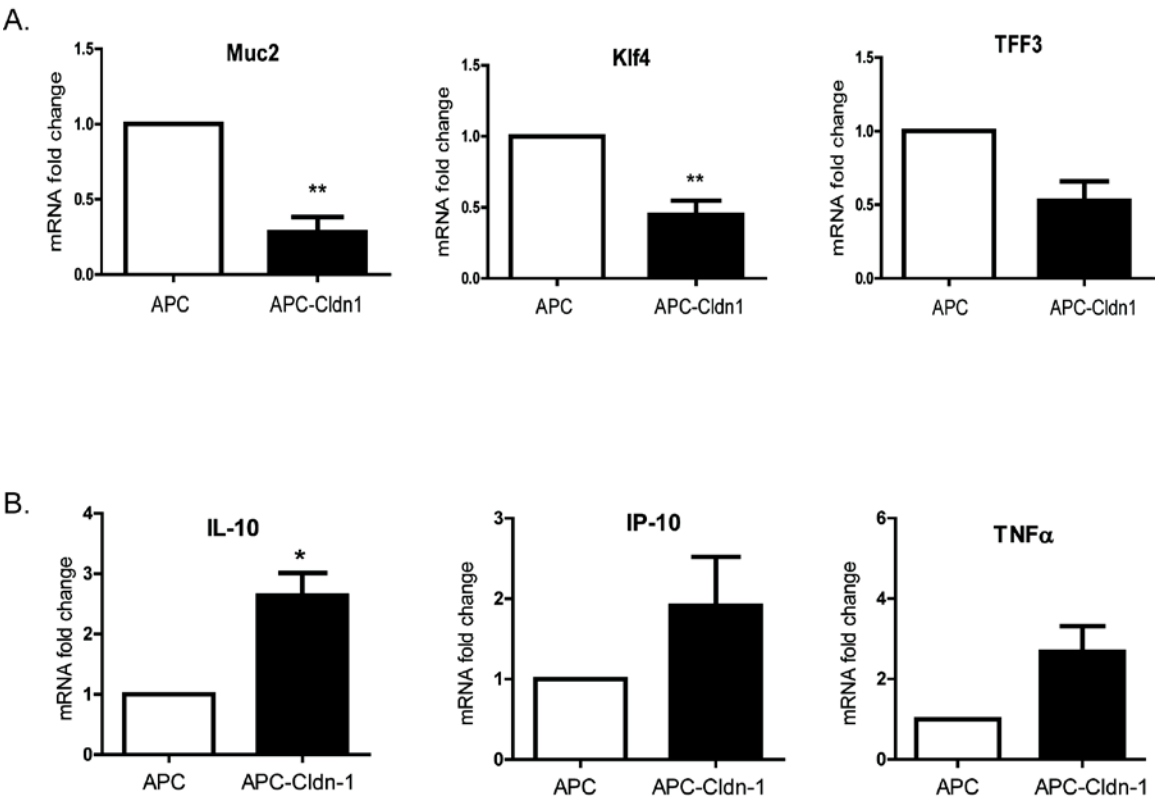


Figure 26. APC-Cldn1 tumors have Decreased Mucosal Defense and Increased Inflammation

(A) qRT-PCR analysis of mucosal defense genes Muc2, Klf4, and Tff3 and (B) inflammatory genes in APC and APC-Cldn1 tumors. *p<0.05, **p<0.005

Claudin-1 increases tumorigenesis in DSS driven model of colon tumorigenesis

Colon tumorigenesis can be induced in APC mice by the administration of the colitis-inducing Dextran sodium sulfate (DSS) (Tanaka et al., 2005). This chemical activates inflammation, which drives the development of colonic tumors in these mice. We have shown previously that DSS exposure in Villin-Cld-1 Tg mice enhance susceptibility to disease. (Pope et al., 2013) Therefore, to observe the effect of claudin-1 overexpression in this model, we treated APC and APC-Cldn1 mice with 2% DSS drinking water for 5 days. Subsequently, mice were given normal drinking water and allowed to recover for 2 weeks until sacrifice. Endoscopic imaging was performed on each mouse on days 5, 8, and 12 following DSS administration. Representative endoscopic images show increased inflammation and tumor development as early as day 5 in APC-Cldn1 mice (Figure 27A), compared to those in APC that developed around day 8. The total number of colonic tumors was quantified for each mouse at the time of sacrifice with APC-Cldn1 mice developing significantly more ($p=0.022$) tumors than APC mice (Figure 27B). Representative H&E images of water and DSS-treated mice for each group are shown (Figure 27C). Although histological analysis of tumors between APC and APC-Cldn1 mice did not differ significantly, we were able to conclude that Claudin-1 overexpression decreased tumor latency and increased tumor number in the DSS-APC model of colon tumorigenesis.

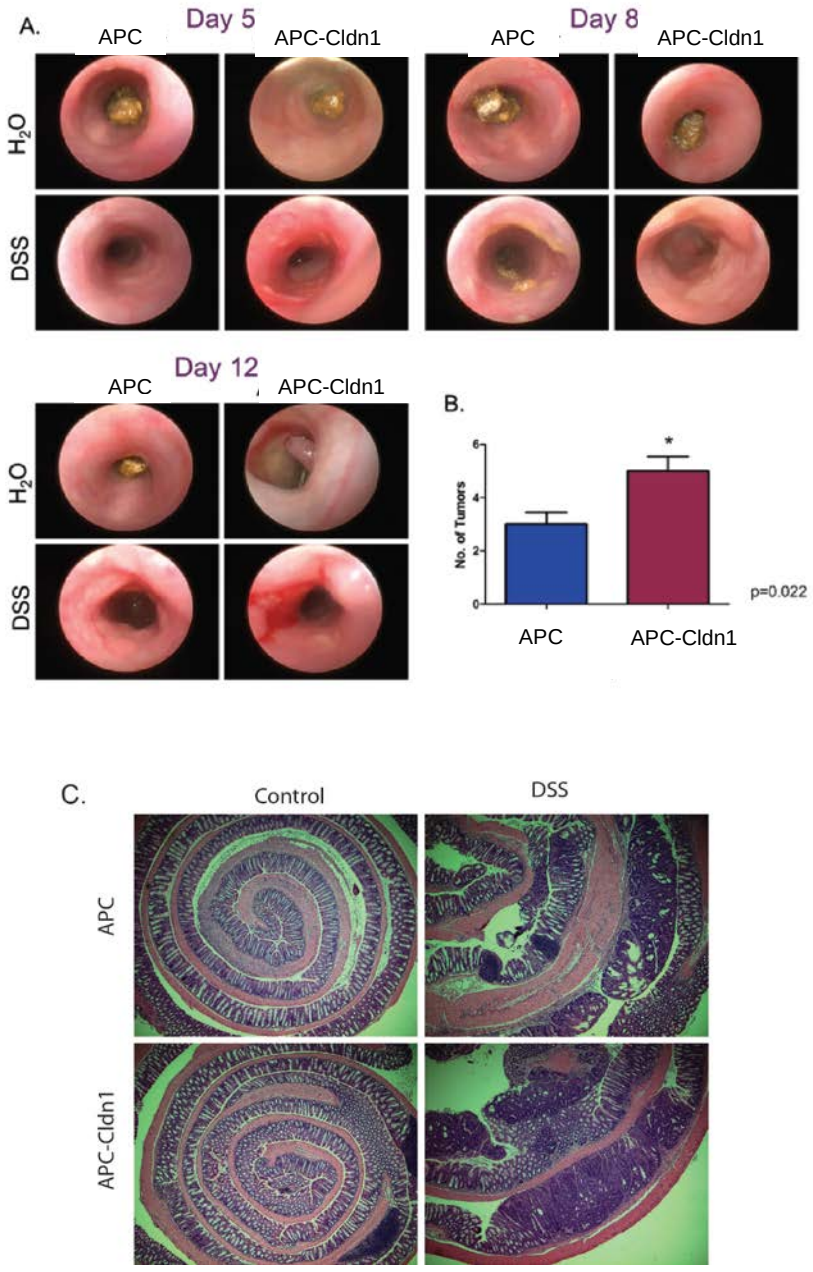


Figure 27. Claudin-1 overexpression increases tumor number in inflammation driven model of tumorigenesis

(A) Endoscopic images of control (water-treated) and treated (2% DSS) mice at the indicated time points, following 7 days of DSS administration. (B) APC-Cldn1 mice developed significantly more colon tumors than APC mice. (C) Representative H&E images. *p<0.05

Claudin-1 increases epithelial permeability, bacterial translocation, and upregulates IL-23 signaling in APC-Cldn1 tumors

In accordance with its classical role, Claudin-1 has been shown to directly alter epithelial permeability (Furuse et al., 2002) (Inai et al., 1999). Additionally, we have shown a baseline increase in colonic epithelial resistance with increased claudin-1 expression (Pope et al., 2013). Therefore, we decided to assess barrier integrity in these mice by measuring permeability to FITC-labeled dextran. APC mice between the ages of 12 to 14 weeks have increased permeability compared to age-matched wild type mice (Puppa et al., 2011). Here, we wanted to determine whether claudin-1 overexpression could increase colonic permeability of APC mice. We observed an increase in permeability to FITC-dextran as early as ten weeks of age in APC-cldn1 mice, with a significant increase (3-fold) observed at 16-18 weeks (Figure 28A), the age at which tumors were found to be present in the colon. Increased permeability in the gut has been frequently linked to increased microbial translocation (Ulluwishewa et al., 2011) (Bergstrom et al., 2010). Using genomic DNA isolated from mice (4 months of age, 3 mice each), we measured translocation via PCR analysis of bacterial specific 16S RNA and observed a significant increase ($p=0.0129$) with claudin-1 overexpression (Figure 28B).

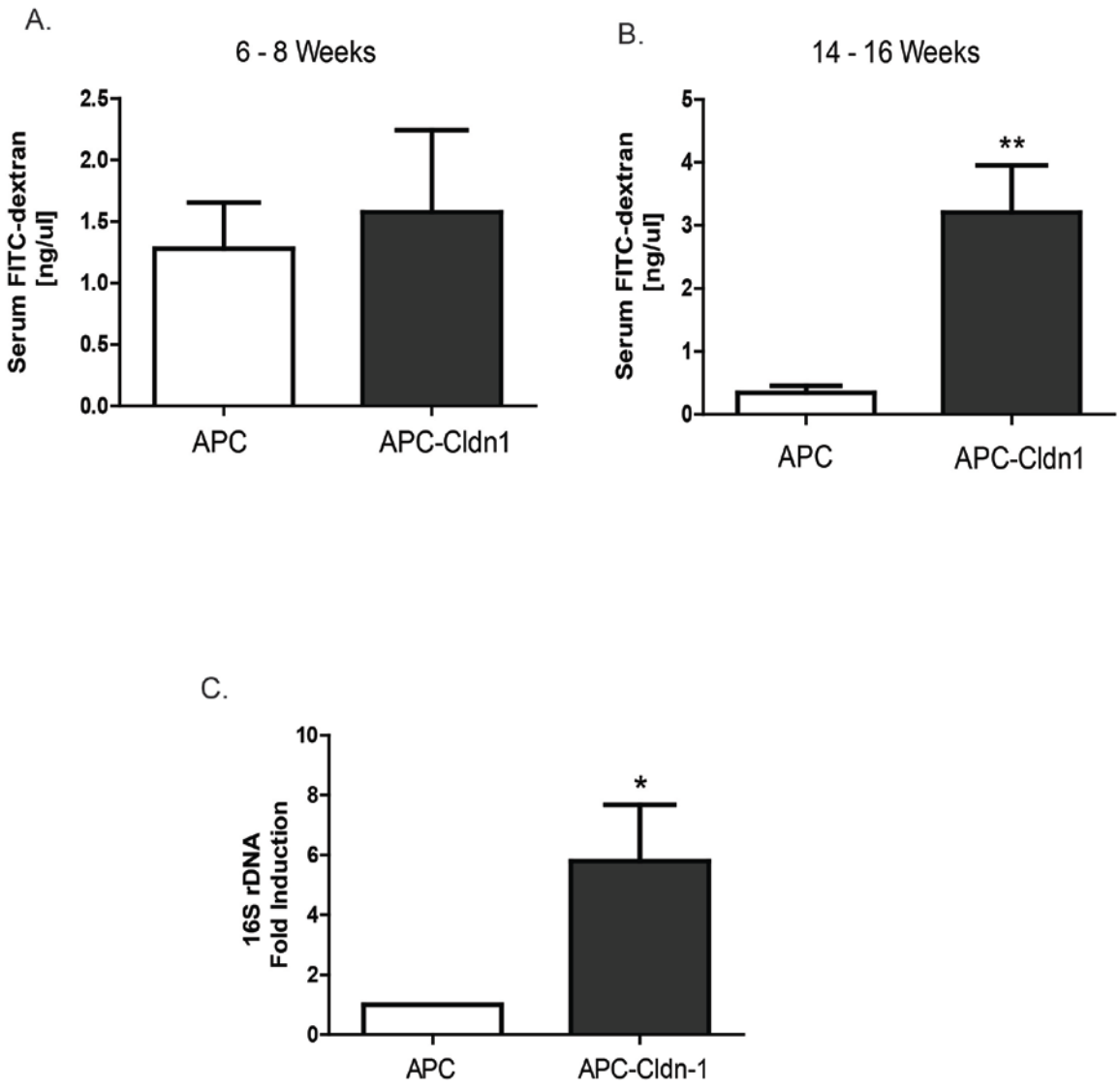


Figure 28. APC-Cldn1 mice have increased colonic permeability and bacterial translocation

(A) Permeability to FITC-dextran via rectal administration at 6-8 weeks (n=5, 6) and (B) 14-16 weeks in APC and APC-Cldn1 mice (n= 6 7). (C) qRT-PCR analysis of 16S rDNA in distal colon of APC and APC-Cldn1 mice at 16 weeks of age (n=3). *p<0.05

Recent studies have shown that increased inflammation, specifically IL-23/IL-17 signaling, contributes to tumor development due to increased permeability and bacterial translocation (Grivennikov et al., 2012). Additionally, presence of IL-23 facilitates bacterial induced colitis (Buonomo et al., 2013). We assessed our model for upregulation of IL-23 signaling, and indeed, microarray analysis showed a significant increase in IL23a cytokine (Figure 25). Validation by qPCR showed a significant increase ($p=0.0022$) in IL23 specific p19 subunit (Figure 29). Additionally, downstream targets IL17A ($p=0.0442$) and IL6 (4-fold induction) were also upregulated in APC-Cldn1 mice. To further analyze IL23 signaling, we performed Immunostaining for phospho-STAT3. Taken together, these results suggests increased claudin-1 expression causes an increase in intestinal permeability in APC mice, thus increasing bacterial translocation. Additionally in response to these changes, IL23 signaling is upregulated.

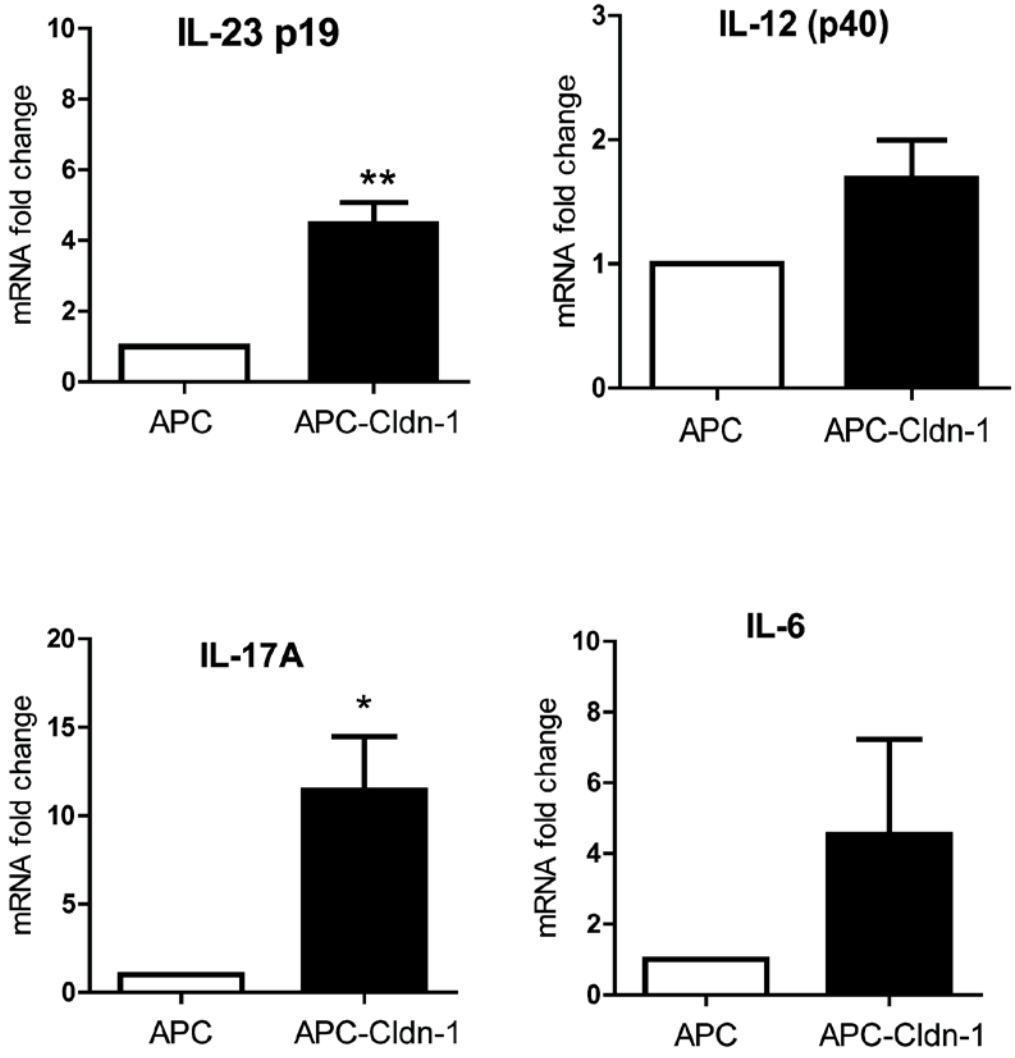


Figure 29. IL-23 signaling is upregulated in tumors of APC-Cldn1 mice

qRT-PCR analysis of IL23 pathway associated genes in APC and APC-Cldn1 mice. *p<0.05, **p<0.005

Discussion

Claudin-1 overexpression has been frequently observed in colon cancer, and mucosal inflammation, however the significance of its upregulation has not been fully studied. We utilized the APCMin model of colon cancer to identify the role of claudin-1 in tumorigenesis and were able to determine that claudin-1 overexpression contributes to tumor growth and progression (Figure 20, Table 3). Not only was there an increase in the amount of tumors, but an increase in size was also observed. As mentioned previously, APC mice do have a limited life span, which is often attributed to the tumor burden of the small intestine and cachexia that develops as a result. As we did not observe a difference in small bowel tumor number, we believe it is the advanced nature of the colonic tumors that contributes to the decreased survival in these mice.

The Wnt and Notch pathways have very important roles in normal colonic development and are known to be dysregulated in disease, most notably colorectal cancer. Claudin-1 is a known target of Tcf/Lef signaling however also seems to participate in potentiation of the pathway as we have seen further upregulation of Wnt signaling in the APC-Cldn1 tumors (Figure 23). This also supports our previously published data of claudin-1 regulating E-cadherin expression through modulation of Wnt/β-catenin activity (Singh et al., 2011). It is of interest to note the Wnt target genes that are upregulated with claudin-1 expression are involved in stem cell maintenance (Figure 23C), as opposed to those that are well known oncogenes, cyclin D1 and c-myc, of which we observed no change in mRNA expression (data not shown). CD44 has been

shown to be a marker of cancer stem cells and responsible for conferring tumorigenic properties to cells (Du et al., 2008), (Zeilstra et al., 2008). Lgr5 is an established intestinal stem cell marker and has also been shown to regulate tumorigenic capacity of colon cancer cells (Barker et al., 2007), (Hirsch et al., 2013). Although not identified as a stem cell marker, Axin2 is an established target of Wnt signaling whose expression has been shown to contribute to colon cancer cell invasion (Wu et al., 2012). Further studies would establish whether claudin-1 could directly regulate expression of these genes.

In previous studies we showed that claudin-1 increases notch activity. We were able to detect increased notch activity as measured by Hes1 and Math1 mRNA expression (Figure 23D). As crosstalk between the Notch and Wnt pathways is not a new concept, it is possible that claudin-1 could rest at the hub of this interaction. The microarray presented several shared genes (Figure 25) between these pathways that were upregulated in tumors from APC-Cldn1 mice. Additionally, Lgr5 expression, which was increased by claudin-1 overexpression, can regulate Notch activity (Hirsch et al., 2013).

We have shown in Cldn-1-Tg mice and colon cancer cell lines that increased claudin-1 expression can decrease expression of mucosal defense genes Muc2, KLF4 and Tff3 (Pope et al., 2013) and in this study microarray data and qPCR analysis confirmed downregulation of these genes in the tumors of APC-Cldn1 mice (Figure 26A). These genes, known to be important in the protection against inflammation and luminal antigens, have also been shown to be important in the protection against tumorigenesis. Klf4 expression can

regulate tumor growth in mouse xenograft studies and tumor number in APC mediated tumorigenesis (Zhang et al., 2006), (Ghaleb et al., 2007). Similarly, Tff3 expression can also regulate tumor growth(Uchino et al., 2000). Muc2 deficient mice, known to develop spontaneous colitis, have been shown to robustly increase colon tumorigenesis when combined with APC mutation (K. Yang et al., 2008). Interestingly, partial loss of Muc2 contributed to tumor development in APC mice in a similar fashion to claudin-1 overexpression. These studies further support Claudin-1 expression can regulate defense gene expression and their loss contributes to claudin-1 mediated tumorigenesis. Additionally this provides further support of a potential role for inflammation. We confirmed increased expression of cytokines commonly upregulated during inflammation in APC-Claudin-1 tumors (Figure 26B).

Sporadic cancer and colitis-associated cancer are frequently studied as two distinct processes separated by their initiating stimulus, loss of APC or inflammatory bowel disease, respectively. Studies, including this work, have highlighted a cooperation of the two, beyond genetic manipulation of specific inflammatory mediators (Grivennikov et al., 2012), (K. Yang et al., 2008), (H. Suzuki et al., 2012), (Chae et al., 2010). This suggests that whereas inflammation is generally thought to be a result of host response to tumor development in “hereditary” development of colon cancer, it may actually function to fuel tumor progression. We employed a model that accelerates the formation of colonic tumors in APC mice to further examine the role of claudin-1 in tumorigenesis (Figure 27). With this model we were able to track tumor

development in both APC and APC-Cldn1 mice in a shorter amount of time. Colon tumors were significantly increased and we also observed that in this model APC-Cldn1 mice developed tumors earlier than APC mice, further confirming our results from the sporadic model (Figure 21).

Loss of mucosal defense genes and the resulting increased inflammation are factors that can be regulated by a breech in barrier dynamics. The role of claudin-1 in the regulation of barrier function was established shortly after its discovery. Additionally, APC mice are inherently more permeable than WT mice. Claudin-1 overexpression was able to enhance permeability (Figure 28A). This change in permeability allows commensal bacteria to freely flow into the lumen of intestinal epithelium. There are studies that show bacteria can contribute to colon tumorigenesis. Specifically, APC mice housed in a germ-free environment produced less tumors than those placed in normal housing conditions(Dove et al., 1997). APC-Cldn1 mice exhibited increased bacterial translocation, further supporting a role for the aid of commensal bacteria in sporadic tumorigenesis.

Recently, studies involving a separate model of Apc Cre-mediated colon tumorigenesis showed that tumor formation was mediated by IL-23 signaling in response to barrier defect and increased bacterial products (Grivennikov et al., 2012). Indeed we observed increased IL-23 and its downstream targets, IL-17 and IL-6 (Figure 29). Since IL-23 signaling is activated in response to bacteria, it is possible in this model that inflammation arises downstream of claudin-1 activation in response to increased bacterial translocation. It is also of interest to note that supernatants of colonic ex vivo explants from Muc2/APC mice had

increased IL-23 secretion (K. Yang et al., 2008). Additionally, many of the genes found to be upregulated in the microarray of APC-Cldn1 mice were also found to be upregulated in the microarray of Muc2/APC mice.

Studies into the role of tight junction proteins in cancer have focused on their role in mechanical aspects of tumorigenesis, i.e., migration and invasion, which is not surprising considering the classical function of these proteins. It is ideal to think of alterations in tight junctions affecting latter stages of tumorigenesis as it relates to loss of polarity and contribution to EMT-like changes, thus facilitating metastasis. Our work shows a causative role of claudin-1 in earlier stages tumorigenesis. We have shown that increased expression of a tight junction protein can increase colonic tumorigenesis in APC mice through IL-23 signaling in response to enhanced permeability and increased bacterial translocation. This observation suggests claudin-1 has an active role in progressing tumorigenesis as opposed to being altered as a result. It is still unclear as to the sequence of events during tumorigenesis in relation to the order of inflammation and claudin-1 upregulation. It is possible to hypothesize a feedback loop may exist that maintains elevated inflammation. Claudin-1 expression has been shown to be regulated by inflammation (Shiozaki et al., 2012),(Weber et al., 2008), and here we have shown that claudin-1 can mediate inflammation through a mechanism involving reduced Muc2 and increased bacterial translocation. Further studies will investigate the specific mechanism by which claudin-1 upregulates IL-23 signaling beyond bacterial upregulation. This study identifies an essential role of increased claudin-1

expression to colon tumorigenesis and provides further insight into the contribution of bacterial-associated inflammation to the development of sporadic colorectal cancer.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Claudin-1 is a key component of the junctional adhesion complex and its contribution to the epithelial barrier has been well established. For the past several years, there have been studies highlighting the dysregulation of claudin-1 in inflammation and cancer. Specifically, claudin-1 expression has been shown to be upregulated in active IBD and in colorectal cancer. However the specific contribution of its increased expression has not been fully elucidated. Therefore, the goal was to determine how claudin-1 overexpression contributes to inflammatory bowel disease and colorectal cancer.

Many signaling pathways and cellular processes involved in development and cell maintenance are commandeered by the cell for use in the progression of disease. In accordance with this fact, I was able to elucidate a novel role for claudin-1 in differentiation of the colonic epithelium. Overexpression of claudin-1 resulted in decreased number of goblet cells and its primary constituent, Muc2 (Figure 8). The implication of this finding proved paramount to the subsequent findings in support of my study, highlighting the role of claudin-1 in inflammation and cancer. Muc2 is a key component of mucosal defense and has been extensively studied in its connection to inflammation and colorectal cancer.

Muc2 deficiency leads to spontaneous colitis (van der Sluis et al., 2006) and genetic combination of Muc2 loss with APC mutation leads to accelerated tumor formation in mice as a result of increased inflammation(K. Yang et al., 2008). Interestingly this effect was found to be dose dependent, as haploinsufficiency of Muc2 produced milder effects in the APC^{Min} mouse compared to total loss. Intestinal overexpression of claudin-1 mimics the effect of partial Muc2 expression, in that mice were able to "normally" develop without overt physiological defects and reach maturity without acquiring spontaneous disease, yet upon stimulus (DSS and APC mutation) was able to enhance susceptibility to disease. Induction of experimental colitis in Cl1-Tg mice caused increased epithelial damage and inflammation compared to WT mice (Figure 10). Additionally, recovery from DSS-induced colitis was impaired in Cl1-Tg mice, as mice were unable to clear the elevated immune response to damage (Figure 13). These studies not only uncovered a role for claudin-1 in the development of IBD, but also a potential role in epithelial repair. As IBD is a chronic disorder, involving repeated cycles of injury and repair, it is important to fully understand the role of claudin-1 in epithelial restitution. *Further studies will seek to confirm involvement of claudin-1 in regenerating epithelium. Specifically, we hope to investigate how increased claudin-1 expression affects epithelial repair, and whether this may prove to be a target of therapy for patients during the active phase of IBD.*

I was also able to identify additional mechanisms and novel interactions in the intestine resulting from claudin-1 overexpression. In line with other studies, claudin-1 overexpression was able to increase proliferation through pERK

signaling and Notch activity (Figure 17) (Kondo et al., 2008). Most significantly, I was able to uncover a mechanism by which claudin-1 regulates Muc2 production and goblet cell differentiation, through activation of Notch signaling through MMP9 (Figure 18). Additionally, elevated Notch signaling was observed in tumors of APC-Cldn1 mice by microarray and qRT-PCR analysis (Figures 23D, 25). Targeting Notch pathway components has proven to be successful in the inhibition of tumor growth and development (Miyamoto et al., 2013). *To this end, pharmacological and/or genetic inhibition of Notch signaling prior to tumor development in APC-Cldn1 mice, or DSS administration in the accelerated* APC^{Min} *model, would help to determine the extent of the role of Notch signaling in tumorigenesis mediated by claudin-1 overexpression.*

Epithelial barrier function is paramount to the maintenance of epithelial homeostasis and the prevention of disease. Increased expression of claudin-1 *in vitro* was shown to illicit an increase in epithelial resistance (Inai et al., 1999). Global depletion of claudin-1 proved detrimental *in vivo*, causing excessive dehydration of the skin (Furuse et al., 2002). Both of these studies were key in establishing the function of claudin-1 in its physiologic role as a tight junction protein. Indeed, as these studies would predict, we observed a baseline increase in the TER with increased claudin-1 expression (Figure 12). But, surprisingly, this was not sufficient to protect against disease. An external stimulus (DSS and APC mutation) was needed to breech barrier function (Figures 12 and 28), and claudin-1 overexpression was able to enhance these changes. Compared to wild type mice, APC mice begin to exhibit increased

permeability as early as 8 weeks of age. By 4 months of age, APC-Cldn1 mice demonstrated nearly a four-fold increase in permeability to FITC-dextran (Figure 28B). It is also at this age where tumors were observed in APC-Cldn1 mice. Indeed, recent studies have shown that colonic tumors, themselves, have increased permeability to FITC-Dextran (Grivennikov et al., 2012), suggesting that the increased permeability in APC-Cldn1 mice may be due to increased tumorigenesis. Additionally, we have shown that claudin-7 expression is altered upon overexpression of claudin-1 (Figure 5). Decreased claudin-7 expression has been linked to defective intestinal architecture which contributed to instances of defective barrier function in older mice, and an increased inflammatory response (Ding et al., 2012). Coincidentally, claudin-7 reduction has also been associated with increased tumorigenesis (Darido et al., 2008), (Lioni et al., 2007). This suggests potential interplay between claudin-7 and claudin-1. *Therefore, we would like to determine whether claudin-7 mediates claudin-1 induced barrier defect. Additionally, claudin-7 loss may also mediate tumor progression in APC-Cldn1 mice.* Of interest, claudin-1 expression has been shown to regulate Zeb-1, which is responsible for transcriptional regulation of E-cadherin (Singh et al., 2011). E-cadherin is one of the major proteins of the adherens junction complex. Loss of E-cadherin is one of the classical markers of cellular transformation, and loss of cell polarity. These processes are also effectors of epithelial barrier integrity.

The contribution of the commensal bacteria in development and progression of disease has been increasingly studied. An increase in bacterial

translocation was observed in APC-Cldn1 mice at 4 months of age, which correlates with tumorigenesis in these mice. Colonic tumors of APC mutant mice were shown to have increased permeability to bacteria via immunostaining (Grivennikov et al., 2012). Additionally, bacteria are able to contribute to tumor growth (Li et al., 2012), barrier defects (Barreau and Hugot, 2014), and inflammation. *We have yet to determine the extent of bacterial translocation in DSS-colitis with claudin-1 overexpression, which would also identify an additional mechanism whereby Cld-1-Tg mice are more susceptibility to disease.*

As mentioned previously, claudin-1 overexpression was able to enhance susceptibility to disease and impair recovery from DSS-colitis. When combined with APC mutation, we also observed an upregulation of inflammatory components and immune regulators via microarray analysis in tumors generated from APC-Cldn1 mice. More importantly we were able to validate increased IL-23 signaling. Studies have identified IL-23/IL-17 signaling as a contributing factor in colon tumorigenesis (Grivennikov et al., 2012), (H. Suzuki et al., 2012). As we have established a connection between increased claudin-1 expression and downregulation of muc2, it is also supportive that tumor explants from APC/Muc2-/- mice had increased IL-23 secretion (K. Yang et al., 2008). IL23 signaling is also upregulated in response to bacteria. It is unclear how increased expression of claudin-1 upregulates IL-23 signaling. *Further studies would seek to identify the mechanism that mediates claudin-1 induced IL-23 signaling. As claudin-1 has been shown to modulate muc2, it is possible that muc2 loss could mediate IL-23 upregulation.*

As we have identified roles for claudin-1 upregulation in colitis and CRC, *we have yet to determine the exact stimulus responsible for the increased expression of claudin-1 and its subsequent mislocalization*. Several studies have provided possible mechanisms however neither have fully elucidated the sequence of events in relation to pathogenesis. Claudin-1 expression has been shown to be upregulated in cancer cell lines by cytokine exposure and contribute to cells tumorigenic capacity (Kondo et al., 2008), (Shiozaki et al., 2012). However, here we have shown that increased expression of claudin-1 may precede increased cytokine upregulation by decreasing mucosal defense. *This suggests there may be a potential feedback loop in which upregulated claudin-1 expression is maintained by inflammation, which is sustained by decreased components of the mucosal barrier.*

Mislocalization of claudin-1 has been observed in active IBD, colitis-associated cancer, and primary tumor and metastatic lesions of spontaneous CRC. Several mechanisms may be at play to facilitate claudin-1 mislocalization. It is possible that an initiating stimulus could cause a change in claudin-1 expression while a separate factor could be responsible in the mislocalization of claudin-1. The relationship between APC and claudin-1 is particularly one of interest that requires further investigation. Previous research in our laboratory has identified an association between claudin-1 upregulation and mutant APC. Additionally, claudin-1 has been shown to associate with Rabs, and additionally during physiological conditions is trafficked to and from the membrane via endosomes. Therefore, it is possible that claudin-1 mislocalization could result in

a trafficking defect. Each of these processes have roles in colon cancer, and therefore may also be exploited in inflammation and cancer to regulate claudin-1.

We and others have proposed claudin-1 to be an ideal therapeutic target in colon cancer and IBD, as genetic manipulation can regulate tumorigenicity of colon cancer cells. Understanding the specific contribution of increased claudin-1 expression would allow us to infer when is the best time for treatment. At least in the context of cancer, increased claudin-1 expression caused tumors to develop earlier and to progress to an advanced diseased state. Through this study, not only were we able to identify roles for claudin-1 in inflammation and CRC, we also identified a novel role for claudin-1 in epithelial homeostasis.

REFERENCES

Alexandre, M.D., Lu, Q., Chen, Y.-H., 2005. Overexpression of claudin-7 decreases the paracellular Cl- conductance and increases the paracellular Na+ conductance in LLC-PK1 cells. Journal of Cell Science 118, 2683–2693.

Araki, Y., Sugihara, H., Hattori, T., 2006. In vitro effects of dextran sulfate sodium on a Caco-2 cell line and plausible mechanisms for dextran sulfate sodium-induced colitis. Oncol. Rep. 16, 1357–1362.

Balda, M.S., Whitney, J.A., Flores, C., González, S., Cereijido, M., Matter, K., 1996. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. The Journal of cell ….

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., Clevers, H., 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449, 1003–1007.

Barreau, F., Hugot, J., 2014. Intestinal barrier dysfunction triggered by invasive bacteria. Curr. Opin. Microbiol. 17C, 91–98.

Ben-Yosef, T., Belyantseva, I.A., Saunders, T.L., Hughes, E.D., Kawamoto, K., Van Itallie, C.M., Beyer, L.A., Halsey, K., Gardner, D.J., Wilcox, E.R., Rasmussen, J., Anderson, J.M., Dolan, D.F., Forge, A., Raphael, Y., Camper, S.A., Friedman, T.B., 2003. Claudin 14 knockout mice, a model for autosomal recessive deafness DFNB29, are deaf due to cochlear hair cell degeneration. Human Molecular Genetics 12, 2049–2061.

Bergstrom, K.S.B., Kissoon-Singh, V., Gibson, D.L., Ma, C., Montero, M., Sham, H.P., Ryz, N., Huang, T., Velcich, A., Finlay, B.B., Chadee, K., Vallance, B.A., 2010. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. PLoS Pathog. 6, e1000902.

Buonomo, E.L., Madan, R., Pramoonjago, P., Li, L., Okusa, M.D., Petri, W.A., 2013. Role of interleukin 23 signaling in Clostridium difficile colitis. J. Infect. Dis. 208, 917–920.

Cassatella, M.A., Gasperini, S., Calzetti, F., Bertagnin, A., Luster, A.D., McDonald, P.P., 1997. Regulated production of the interferon-gamma-inducible protein-10 (IP-10) chemokine by human neutrophils. Eur. J. Immunol. 27, 111–115.

Chae, W.-J., Gibson, T.F., Zelterman, D., Hao, L., Henegariu, O., Bothwell, A.L.M., 2010. Ablation of IL-17A abrogates progression of spontaneous intestinal tumorigenesis. Proc. Natl. Acad. Sci. U.S.A. 107, 5540–5544.

Chichlowski, M., Hale, L.P., 2008. Bacterial-mucosal interactions in inflammatory bowel disease: an alliance gone bad. Am. J. Physiol. Gastrointest. Liver Physiol. 295, G1139–49.

Coant, N., Mkaddem, S.B., Pedruzzi, E., Guichard, C., Treton, X., Ducroc, R., Freund, J.-N., Cazals-Hatem, D., Bouhnik, Y., Woerther, P.-L., Skurnik, D., Grodet, A., Fay, M., Biard, D., Lesuffleur, T., Deffert, C., Moreau, R., Groyer, A., Krause, K.-H., Daniel, F., Ogier-Denis, E., 2010. NADPH Oxidase 1 Modulates WNT and NOTCH1 Signaling To Control the Fate of Proliferative Progenitor Cells in the Colon. Molecular and Cell Biology 30, 2636–2650.

Dahan, S., Rabinowitz, K.M., Martin, A.P., Berin, M.C., Unkeless, J.C., Mayer, L., 2011. Notch-1 signaling regulates intestinal epithelial barrier function, through interaction with CD4+ T cells, in mice and humans. Gastroenterology 140, 550–559.

Darido, C., Buchert, M., Pannequin, J., Bastide, P., Zalzali, H., Mantamadiotis, T., Bourgaux, J.F., Garambois, V., Jay, P., Blache, P., Joubert, D., Hollande, F., 2008. Defective Claudin-7 Regulation by Tcf-4 and Sox-9 Disrupts the Polarity and Increases the Tumorigenicity of Colorectal Cancer Cells. Cancer Research 68, 4258–4268.

Dhawan, P., Singh, A.B., Deane, N.G., No, Y., Shiou, S.-R., Schmidt, C., Neff, J., Washington, M.K., Beauchamp, R.D., 2005. Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. J. Clin. Invest. 115, 1766–1776.

Ding, L., Lu, Z., Foreman, O., Tatum, R., Lu, Q., Renegar, R., Cao, J., Chen, Y.-H., 2012. Inflammation and disruption of the mucosal architecture in claudin-7-deficient mice. Gastroenterology 142, 305–315.

Dove, W.F., Clipson, L., Gould, K.A., Luongo, C., Marshall, D.J., Moser, A.R., Newton, M.A., Jacoby, R.F., 1997. Intestinal neoplasia in the ApcMin mouse: independence from the microbial and natural killer (beige locus) status. Cancer Research 57, 812–814.

Du, L., Wang, H., He, L., Zhang, J., Ni, B., Wang, X., Jin, H., Cahuzac, N., Mehrpour, M., Lu, Y., Chen, Q., 2008. CD44 is of functional importance for colorectal cancer stem cells. Clin. Cancer Res. 14, 6751–6760.

Evans, M.J., Hahn, von, T., Tscherne, D.M., Syder, A.J., Panis, M., Wölk, B., Hatziioannou, T., McKeating, J.A., Bieniasz, P.D., Rice, C.M., 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 446, 801–805.

Fang, Y.S., Zhu, L.M., Sun, Z.G., Yu, L.Z., Xu, H., 2012. 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. 44, 993–995.

Fre, S., Hannezo, E., Sale, S., Huyghe, M., Lafkas, D., Kissel, H., Louvi, A., Greve, J., Louvard, D., Artavanis-Tsakonas, S., 2011. Notch lineages and activity in intestinal stem cells determined by a new set of knock-in mice. PLoS ONE 6, e25785.

Fre, S., Pallavi, S.K., Huyghe, M., Laé, M., Janssen, K.-P., Robine, S., Artavanis-Tsakonas, S., Louvard, D., 2009. Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. Proc. Natl. Acad. Sci. U.S.A. 106, 6309–6314.

Fu, J., Wei, B., Wen, T., Johansson, M.E.V., Liu, X., Bradford, E., Thomsson, K.A., McGee, S., Mansour, L., Tong, M., McDaniel, J.M., Sferra, T.J., Turner, J.R., Chen, H., Hansson, G.C., Braun, J., Xia, L., 2011. Loss of intestinal core 1-derived O-glycans causes spontaneous colitis in mice. J. Clin. Invest. 121, 1657–1666.

Furuse, M., 1998. Claudin-1 and -2: Novel Integral Membrane Proteins Localizing at Tight Junctions with No Sequence Similarity to Occludin. The Journal of Cell Biology 141, 1539–1550.

Furuse, M., Hata, M., Furuse, K., Yoshida, Y., Haratake, A., Sugitani, Y., Noda, T., Kubo, A., Tsukita, S., 2002. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. Journal of Cell Biology 156, 1099–1111.

Garg, P., Jeppsson, S., Dalmasso, G., Ghaleb, A.M., Mcconnell, B.B., Yang, V.W., Gewirtz, A.T., Merlin, D., Sitaraman, S.V., 2011. Notch1 regulates the effects of matrix metalloproteinase-9 on colitis-associated cancer in mice. Gastroenterology 141, 1381–1392.

Garg, P., Ravi, A., Patel, N.R., Roman, J., Gewirtz, A.T., Merlin, D., Sitaraman, S.V., 2007. Matrix metalloproteinase-9 regulates MUC-2 expression through its effect on goblet cell differentiation. Gastroenterology 132, 1877–1889.

Ghaleb, A.M., McConnell, B.B., Nandan, M.O., Katz, J.P., Kaestner, K.H., Yang, V.W., 2007. Haploinsufficiency of Kruppel-Like Factor 4 Promotes Adenomatous Polyposis Coli Dependent Intestinal Tumorigenesis. Cancer Research 67, 7147–7154.

Gow, A., Davies, C., Southwood, C.M., Frolenkov, G., Chrustowski, M., Ng, L., Yamauchi, D., Marcus, D.C., Kachar, B., 2004. Deafness in Claudin 11-null mice reveals the critical contribution of basal cell tight junctions to stria vascularis function. J. Neurosci. 24, 7051–7062.

Grivennikov, S.I., Wang, K., Mucida, D., Stewart, C.A., Schnabl, B., Jauch, D., Taniguchi, K., Yu, G.-Y., Österreicher, C.H., Hung, K.E., Datz, C., Feng, Y., Fearon, E.R., Oukka, M., Tessarollo, L., Coppola, V., Yarovinsky, F., Cheroutre, H., Eckmann, L., Trinchieri, G., Karin, M., 2012. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. Nature 491, 254–258.

Hasnain, S.Z., Wang, H., Ghia, J.E., Haq, N., Deng, Y., Velcich, A., Grencis, R.K., Thornton, D.J., Khan, W.I., 2010. Mucin Gene Deficiency in Mice Impairs Host Resistance to an Enteric Parasitic Infection. Gastroenterology 138, 1763–1771.e5.

Hirsch, D., Barker, N., McNeil, N., Hu, Y., Camps, J., McKinnon, K., Clevers, H., Ried, T., Gaiser, T., 2013. LGR5 positivity defines stem-like cells in colorectal cancer. Carcinogenesis.

Hogan, M.B., Peele, K., Wilson, N.W., 2012. Skin barrier function and its importance at the start of the atopic march. J Allergy (Cairo) 2012, 901940.

Hollingsworth, M.A., Swanson, B.J., 2004. Mucins in cancer: protection and control of the cell surface. Nat. Rev. Cancer 4, 45–60.

Huo, Q., Kinugasa, T., Wang, L., Huang, J., Zhao, J., Shibaguchi, H., Kuroki, M., Tanaka, T., Yamashita, Y., Nabeshima, K., Iwasaki, H., Kuroki, M., 2009. Claudin-1 protein is a major factor involved in the tumorigenesis of colorectal cancer. Anticancer Res. 29, 851–857.

Ikari, A., Takiguchi, A., Atomi, K., Sato, T., Sugatani, J., 2011. Decrease in claudin-2 expression enhances cell migration in renal epithelial madin-darby canine kidney cells. J. Cell. Physiol. 226, 1471–1478.

Inai, T., Kobayashi, J., Shibata, Y., 1999. Claudin-1 contributes to the epithelial barrier function in MDCK cells. Eur. J. Cell Biol. 78, 849–855.

Johansson, M.E.V., Phillipson, M., Petersson, J., Velcich, A., Holm, L., Hansson, G.C., 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc. Natl. Acad. Sci. U.S.A. 105, 15064–15069.

Kinugasa, T., Akagi, Y., Yoshida, T., Ryu, Y., Shiratuchi, I., Ishibashi, N., Shirouzu, K., 2010. Increased claudin-1 protein expression contributes to tumorigenesis in ulcerative colitis-associated colorectal cancer. Anticancer Res. 30, 3181–3186.

Kondo, J., Sato, F., Kusumi, T., Liu, Y., Motonari, O., Sato, T., Kijima, H., 2008. Claudin-1 expression is induced by tumor necrosis factor-α in human pancreatic cancer cells. Int. J. Mol. Med. 22, 645–649.

Kufe, D.W., 2009. Mucins in cancer: function, prognosis and therapy. Nat. Rev. Cancer 9, 874–885.

Laukoetter, M.-G., Nava, P., Nusrat, A., 2008. Role of the intestinal barrier in inflammatory bowel disease. WJG 14, 401.

Li, Y., Kundu, P., Seow, S.W., de Matos, C.T., Aronsson, L., Chin, K.C., Karre, K., Pettersson, S., Greicius, G., 2012. Gut microbiota accelerate tumor growth via c-jun and STAT3 phosphorylation in APCMin/+ mice. Carcinogenesis 33, 1231–1238.

Lioni, M., Brafford, P., Andl, C., Rustgi, A., El-Deiry, W., Herlyn, M., Smalley, K.S.M., 2007. Dysregulation of Claudin-7 Leads to Loss of E-Cadherin Expression and the Increased Invasion of Esophageal Squamous Cell Carcinoma Cells. The American Journal of Pathology 170, 709–721.

Maloy, K.J., Powrie, F., 2011. Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature 474, 298–306.

Marjou, el, F., Janssen, K.-P., Chang, B.H.-J., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D., Robine, S., 2004. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 39, 186–193.

McCart, A.E., Vickaryous, N.K., Silver, A., 2008. Apc mice: Models, modifiers and mutants. Pathology - Research and Practice 204, 479–490.

McGuckin, M.A., Lindén, S.K., Sutton, P., Florin, T.H., 2011. Mucin dynamics and enteric pathogens. Nat. Rev. Microbiol. 9, 265–278.

Mittal, S., Subramanyam, D., Dey, D., Kumar, R.V., Rangarajan, A., 2009. Cooperation of Notch and Ras/MAPK signaling pathways in human breast carcinogenesis. Mol Cancer 8, 128.

Miwa, N., Furuse, M., Tsukita, S., Niikawa, N., Nakamura, Y., Furukawa, Y., 2001. Involvement of Claudin-1 in the beta-CateninTcf Signaling Pathway and its Frequent Upregulation in Human Colorectal Cancers. Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics, 12 11, 469–476.

Miyamori, H., Takino, T., Kobayashi, Y., Tokai, H., Itoh, Y., Seiki, M., Sato, H., 2001. Claudin promotes activation of pro-matrix metalloproteinase-2 mediated by membrane-type matrix metalloproteinases. J. Biol. Chem. 276, 28204–28211.

Miyamoto, S., Nakanishi, M., Rosenberg, D.W., 2013. Suppression of colon carcinogenesis by targeting Notch signaling. Carcinogenesis 34, 2415–2423.

Morin, P.J., 2005. Claudin Proteins in Human Cancer: Promising New Targets for Diagnosis and Therapy. Cancer Research 65, 9603–9606.

Muto, S., Hata, M., Taniguchi, J., Tsuruoka, S., Moriwaki, K., Saitou, M., Furuse, K., Sasaki, H., Fujimura, A., Imai, M., Kusano, E., Tsukita, S., Furuse, M., 2010. Claudin-2-deficient mice are defective in the leaky and cation-selective paracellular permeability properties of renal proximal tubules. Proc. Natl. Acad. Sci. U.S.A. 107, 8011–8016.

Műzes, G., Molnár, B., Tulassay, Z., Sipos, F., 2012. Changes of the cytokine profile in inflammatory bowel diseases. WJG 18, 5848–5861.

Nakamura, T., Tsuchiya, K., Watanabe, M., 2007. Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. J Gastroenterol 42, 705–710.

Niessen, C.M., 2007. Tight Junctions/Adherens Junctions: Basic Structure and Function. J Investig Dermatol 127, 2525–2532.

Okamoto, R., 2011. Epithelial regeneration in inflammatory bowel diseases. Inflammation and Regeneration 31, 275–281.

Okamoto, R., Tsuchiya, K., Nemoto, Y., Akiyama, J., Nakamura, T., Kanai, T., Watanabe, M., 2009. Requirement of Notch activation during regeneration of the intestinal epithelia. Am. J. Physiol. Gastrointest. Liver Physiol. 296, G23–35.

Oku, N., Sasabe, E., Ueta, E., Yamamoto, T., Osaki, T., 2006. 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 membrane-type MMP-1. Cancer Research 66, 5251–5257.

Oliveira, S.S., Morgado-Diaz, J.A., 2007. Claudins: multifunctional players in epithelial tight junctions and their role in cancer. Cell. Mol. Life Sci. 64, 17–28.

Peignon, G., Durand, A., Cacheux, W., Ayrault, O., Terris, B., Laurent-Puig, P., Shroyer, N.F., van Seuningen, I., Honjo, T., Perret, C., Romagnolo, B., 2011. Complex interplay between β-catenin signalling and Notch effectors in intestinal tumorigenesis. Gut 60, 166–176.

Pope, J.L., Bhat, A.A., Sharma, A., Ahmad, R., Krishnan, M., Washington, M.K., Beauchamp, R.D., Singh, A.B., Dhawan, P., 2013. Claudin-1 regulates intestinal epithelial homeostasis through the modulation of Notch-signalling. Gut. Published Online First: 13 June 2013. *doi:10.1136/gutjnl-2012-304241*

Puppa, M.J., White, J.P., Sato, S., Cairns, M., Baynes, J.W., Carson, J.A., 2011. Gut barrier dysfunction in the ApcMin/+ mouse model of colon cancer cachexia. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1812, 1601–1606.

Qiao, L., Wong, B.C.Y., 2009. Role of Notch signaling in colorectal cancer. Carcinogenesis 30, 1979–1986.

Reedijk, M., Odorcic, S., Zhang, H., Chetty, R., Tennert, C., Dickson, B.C., Lockwood, G., Gallinger, S., Egan, S.E., 2008. Activation of Notch signaling in human colon adenocarcinoma. Int J Oncol 33, 1223–1229.

Resnick, M.B., Konkin, T., Routhier, J., Sabo, E., Pricolo, V.E., 2005. Claudin-1 is a strong prognostic indicator in stage II colonic cancer: a tissue microarray study. Modern Pathology 18, 511–518.

Sawada, N., 2013. Tight junction-related human diseases. Pathol. Int. 63, 1–12.

Sawey, E.T., Johnson, J.A., Crawford, H.C., 2007. Matrix metalloproteinase 7 controls pancreatic acinar cell transdifferentiation by activating the Notch signaling pathway. Proc. Natl. Acad. Sci. U.S.A. 104, 19327–19332.

Schmitz, H., Barmeyer, C., Fromm, M., Runkel, N., Foss, H.-D., Bentzel, C.J., Riecken, E.-O., Schulzke, J.-D., 1999. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis.

Gastroenterology 116, 301–309.
Shin, K., Fogg, V.C., Margolis, B., 2006. Tight Junctions and Cell Polarity. Annu. Rev. Cell Dev. Biol. 22, 207–235.

Shiozaki, A., Bai, X.-H., Shen-Tu, G., Moodley, S., Takeshita, H., Fung, S.-Y., Wang, Y., Keshavjee, S., Liu, M., 2012. Claudin 1 mediates TNFα-induced gene expression and cell migration in human lung carcinoma cells. PLoS ONE 7, e38049.

Sikandar, S.S., Pate, K.T., Anderson, S., Dizon, D., Edwards, R.A., Waterman, M.L., Lipkin, S.M., 2010. 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 70, 1469–1478.

Singh, A.B., Sharma, A., Dhawan, P., 2010. Claudin family of proteins and cancer: an overview. J Oncol 2010, 541957.

Singh, A.B., Sharma, A., Dhawan, P., 2012. Claudin-1 expression confers resistance to anoikis in colon cancer cells in a Src-dependent manner. Carcinogenesis 33, 2538–2547.

Singh, A.B., Sharma, A., Smith, J.J., Krishnan, M., Chen, X., Eschrich, S., Washington, M.K., Yeatman, T.J., Beauchamp, R.D., Dhawan, P., 2011. Claudin-1 up-regulates the repressor ZEB-1 to inhibit E-cadherin expression in colon cancer cells. Gastroenterology 141, 2140–2153.

Sonoshita, M., Aoki, M., Fuwa, H., Aoki, K., Hosogi, H., Sakai, Y., Hashida, H., Takabayashi, A., Sasaki, M., Robine, S., Itoh, K., Yoshioka, K., Kakizaki, F., Kitamura, T., Oshima, M., Taketo, M.M., 2011. Suppression of colon cancer metastasis by Aes through inhibition of Notch signaling. Cancer Cell 19, 125–137.

Su, L., Shen, L., Clayburgh, D.R., Nalle, S.C., Sullivan, E.A., Meddings, J.B., Abraham, C., Turner, J.R., 2009. Targeted Epithelial Tight Junction Dysfunction Causes Immune Activation and Contributes to Development of Experimental Colitis. Gastroenterology 136, 551–563.

Suh, Y., Yoon, C.-H., Kim, R.-K., Lim, E.-J., Oh, Y.S., Hwang, S.-G., An, S., Yoon, G., Gye, M.C., Yi, J.-M., Kim, M.-J., Lee, S.-J., 2012. Claudin-1 induces epithelial-mesenchymal transition through activation of the c-Abl-ERK signaling pathway in human liver cells. Oncogene.

Sundaram, M.V., 2005. The love-hate relationship between Ras and Notch. Genes & Development 19, 1825–1839.

Suzuki, H., Ogawa, H., Miura, K., Haneda, S., Watanabe, K., Ohnuma, S., Sasaki, H., Sase, T., Kimura, S., Kajiwara, T., Komura, T., Toshima, M., Matsuda, Y., Shibata, C., Sasaki, I., 2012. IL-23 directly enhances the proliferative and invasive activities of colorectal carcinoma. Oncol Lett 4, 199–204.

Suzuki, T., Yoshinaga, N., Tanabe, S., 2011. Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. Journal of Biological Chemistry 286, 31263–31271.

Tanaka, T., Kohno, H., Suzuki, R., Hata, K., Sugie, S., Niho, N., Sakano, K., Takahashi, M., Wakabayashi, K., 2005. Dextran sodium sulfate strongly promotes colorectal carcinogenesis inApcMin/+ mice: Inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. Int. J. Cancer 118, 25–34.

Tedelind, S., Ericson, L., Karlsson, J., 2003. Interferon-gamma down-regulates claudin-1 and impairs the epithelial barrier function in primary cultured human thyrocytes. … journal of endocrinology.

Tsukita, S., Furuse, M., 1998. Overcoming barriers in the study of tight junction functions: from occludin to claudin. Genes Cells 3, 569–573.

Tsukita, S., Yamazaki, Y., Katsuno, T., Tamura, A., Tsukita, S., 2008. Tight junction-based epithelial microenvironment and cell proliferation. Oncogene 27, 6930–6938.

Turner, J.R., 2009. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol 9, 799–809.

Tytgat, K.M., van der Wal, J.W., Einerhand, A.W., Büller, H.A., Dekker, J., 1996. Quantitative analysis of MUC2 synthesis in ulcerative colitis. Biochemical and Biophysical Research Communications 224, 397–405.

Uchino, H., Kataoka, H., Itoh, H., Hamasuna, R., Koono, M., 2000. Overexpression of intestinal trefoil factor in human colon carcinoma cells reduces cellular growth in vitro and in vivo. Gastroenterology.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., Roy, N.C., 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. J. Nutr. 141, 769–776.

van der Flier, L.G., Clevers, H., 2009. Stem Cells, Self-Renewal, and Differentiation in the Intestinal Epithelium. Annu. Rev. Physiol. 71, 241–260.

van der Sluis, M., De Koning, B.A.E., De Bruijn, A.C.J.M., Velcich, A., Meijerink, J.P.P., van Goudoever, J.B., Buller, H.A., Dekker, J., van Seuningen, I., Renes, I.B., Einerhand, A.W.C., 2006. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 131, 117–129.

VanDussen, K.L., Carulli, A.J., Keeley, T.M., Patel, S.R., Puthoff, B.J., Magness, S.T., Tran, I.T., Maillard, I., Siebel, C., Kolterud, Å., Grosse, A.S., Gumucio, D.L., Ernst, S.A., Tsai, Y.-H., Dempsey, P.J., Samuelson, L.C., 2012. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. Development 139, 488–497.

Vooijs, M., Liu, Z., Kopan, R., 2011. Notch: architect, landscaper, and guardian of the intestine. Gastroenterology 141, 448–459.

Weber, C.R., Nalle, S.C., Tretiakova, M., Rubin, D.T., Turner, J.R., 2008. Claudin-1 and claudin-2 expression is elevated in inflammatory bowel disease and may contribute to early neoplastic transformation. Lab Invest 88, 1110–1120.

Wu, Z.-Q., Brabletz, T., Fearon, E., Willis, A.L., Hu, C.Y., Li, X.-Y., Weiss, S.J., 2012. Canonical Wnt suppressor, Axin2, promotes colon carcinoma oncogenic activity. Proc. Natl. Acad. Sci. U.S.A. 109, 11312–11317.

Yang, K., Popova, N.V., Yang, W.C., Lozonschi, I., Tadesse, S., Kent, S., Bancroft, L., Matise, I., Cormier, R.T., Scherer, S.J., Edelmann, W., Lipkin, M., Augenlicht, L., Velcich, A., 2008. Interaction of Muc2 and Apc on Wnt Signaling and in Intestinal Tumorigenesis: Potential Role of Chronic Inflammation. Cancer Research 68, 7313–7322.

Yang, W., Velcich, A., Lozonschi, I., Liang, J., Nicholas, C., Zhuang, M., Bancroft, L., Augenlicht, L.H., 2005. Inactivation of p21WAF1/cip1 enhances intestinal tumor formation in Muc2-/- mice. The American Journal of Pathology 166, 1239–1246.

Yeruva, S., Ramadori, G., Raddatz, D., 2008. 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 23, 305–317.

Zavala-Zendejas, V.E., Torres-Martinez, A.C., 2011. Claudin-6, 7, or 9 Overexpression in the Human Gastric Adenocarcinoma Cell Line AGS Increases Its Invasiveness, Migration, and Proliferation Rate, Cancer Investigation, Informa Healthcare. Cancer.

Zeilstra, J., Joosten, S.P.J., Dokter, M., Verwiel, E., Spaargaren, M., Pals, S.T., 2008. Deletion of the WNT Target and Cancer Stem Cell Marker CD44 in Apc(Min/+) Mice Attenuates Intestinal Tumorigenesis. Cancer Research 68, 3655–3661.

Zeissig, S., Bürgel, N., Günzel, D., Richter, J., Mankertz, J., Wahnschaffe, U., Kroesen, A.J., Zeitz, M., Fromm, M., Schulzke, J.-D., 2007. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. Gut 56, 61–72.

Zhang, W., Chen, X., Kato, Y., Evans, P., 2006. Novel Cross Talk of Krüppel-Like Factor 4 and β-Catenin Regulates Normal Intestinal Homeostasis and Tumor Repression. … and cellular biology.

Zheng, X., Tsuchiya, K., Okamoto, R., Iwasaki, M., Kano, Y., Sakamoto, N., Nakamura, T., Watanabe, M., 2011. Suppression of hath1 gene expression directly regulated by hes1 via notch signaling is associated with goblet cell depletion in ulcerative colitis. Inflamm Bowel Dis 17, 2251–2260.