ABSTRACT

JIN, YOUNGGEON. Regulation of the Intestinal Barrier in Digestive Disease. (Under the direction of Dr. Anthony Blikslager.)

The gastrointestinal barrier has a critical role in the regulation of mucosal homeostasis. The tight junction is the apical-most constituent of the intercellular junctional complex and provides a mucosal barrier in several physiological and pathophysiological conditions. We have previously reported that the ClC-2 chloride channel has an important role in the regulation of tight junction barrier function during experimental colitis. We have found that the pharmaceutical ClC-2 chloride channel agonist lubiprostone initiates intestinal barrier repair in ischemic-injured intestine. We studied the role of lubiprostone in the regulation of tight junctions in murine colitis models. We generated murine colitis models using dextran sulfate sodium (DSS) or 2, 4, 5-trinitrobenxene sulfonic acid (TNBS) in wild type ClC-$2^{-/-}$ mice. Orally administered lubiprostone significantly reduced the severity of colitis and reduced intestinal permeability in mice colitis models. Preventive administration of lubiprostone resulted in a dramatic recovery of tight junction proteins, decreased crypt proliferation, and increased intestinal epithelial differentiation in mice with DSS-induced colitis. These results suggest that pharmaceutical ClC-2 activation can be used clinically to maintain intestinal barrier homeostasis in patients with inflammatory bowel disease.

Additionally, we investigated the mechanism of tight junction redistribution in an *in vitro* hypoxia/reoxygenation (H/R) model. We hypothesized that MLCK-dependent redistribution of tight junction proteins might be an essential component of epithelial barrier maintenance during H/R injury in Caco-2BBe human intestinal cells. We induced H/R injury

with 95% N_2/5% CO_2 for 2 hours and measured fluorescent-dextran flux at various time points during the reoxygenation. H/R injury causes increased paracellular permeability of 4 kDa FITC-dextran accompanied by redistribution of the tight junction protein occludin as well as increased MLC phosphorylation. Our study has also shown that MLCK inhibition attenuates H/R injury-induced paracellular barrier disruption. It is suggested that MLCK-dependent MLC phosphorylation may be a critical mechanism in the tight junction barrier breakdown caused by H/R injury.

We conclude that regulation of the intestinal tight junction barrier is critical for management of digestive disease. Most importantly, we believe that ClC-2 and MLCK may be therapeutically effective in patients with intestinal barrier disorder-associated disease.

Regulation of the Intestinal Barrier in Digestive Disease

by
Younggeon Jin

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina

2014

APPROVED BY:

_____	_____
Dr. Anthony T. Blikslager Chair of Advisory Committee	Dr. Adam Moeser
_____	_____
Dr. Jun Ninomiya-Tsuji	Dr. Sam L. Jones

BIOGRAPHY

Younggeon Jin was born on February 23, 1982, in Jeju, South Korea. He grew up with his parents Shin-Hyun Jin and Sunna Kim, and his sisters Youngrim Jin, Jungrim Jin, Jungbin Jin, and Juyoun Jin. He completed his Doctorate of Veterinary Medicine from the College of Veterinary Medicine at Jeju National University in Feb 2009. He worked at the Samsung Biomedical Research Institute for two and a half years before joining North Carolina State University for PhD study in August 2011. His PhD graduate work in gastrointestinal pathophysiology was performed under the direction of Dr. Anthony Blikslager.

ACKNOWLEDGMENTS

There are several people whom I would like to thank for their guidance, advice, and friendship offered throughout my PhD. Degree at North Carolina State University. First of all, I sincerely thank Dr. Anthony Blikslager for providing me this wonderful opportunity of graduate research with the mentorship and opportunities to develop into a successful researcher. I would also like to thank Drs. Sam Jones, Adam Moeser, and Jun Ninomiya-Tsuji for agreeing to serve as committee members and their valuable advice throughout my degree. I would like to express my deepest gratitude to my fellow laboratory colleagues Dr. Liara Gonzalez, Tiffany Pridgen, Ashwini Poopal, Mary Brown, Vassili kouprianov and Bailey Brame for their support and help with my research studies. I thank the staff at Laboratory Animal Resources and Histopathology Laboratory for their excellent technical advice and support. Dr. Troy Ghashghaei helped us with confocal microscopy.

Above all I would like to thank my wife Joohee Kang and children Audrey and Austin for their personal support and patience at all times. I am grateful to my family, especially my parents Mr. Shin-Hyun Jin and Ms. Sunna Kim for their support. I would also like to thank my parents-in-law Kui-Ok Kang and Duckjin Park for their support and inspiration.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 2.

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

ROLE OF TIGHT JUNCTIONS IN THE REGULATION OF INTESTINAL BARRIER FUNCTION

INTRODUCTION

The tight junction forms the apical-most constituent of the intercellular junctional complex, serving as an epithelial barrier, which is not absolute but is selectively permeable. The tight junctions consist of transmembrane, cytoskeletal, and signaling proteins with complex interactions. Tight junction proteins include occludin, claudins, junctional adhesion molecules (JAMs), and zonula occludens proteins (ZOs), and their interaction regulates tight junction structure and function. Intestinal tight junctions between epithelial cells maintain the intestinal barrier while regulating paracellular permeability of ions, nutrients, and water. The regulation of tight junction barrier function by cytokines and growth factors has recently been highlighted. The study of these interactions is crucial for the understanding of the progression of several intestinal disorders including inflammatory bowel disease (IBD), ischemic injury, and irritable bowel syndrome (IBS). Further studies regarding the regulation of tight junctions will lead to a better understanding of the molecular mechanisms that regulate barrier function in physiological and pathophysiological conditions.

1. The role of tight junctions in epithelial barrier function

Tight junctions are the apical-most constituent of the intercellular junctional complex which also includes intermediate junctions, desmosomes, and gap junctions (1). They have two functions: gate function and fence function. Fence function refers to the ability of tight junctions to restrict the movement of lipids and membrane proteins between the apical and the basolateral membrane, whereas gate function refers to regulation of passive diffusion of solutes and macromolecules thorough the interepithelial space (2).

The movement of nutrients and water across epithelium occurs through both transcellular and paracellular pathways. The transcellular pathway is associated with active or passive movement of water and solutes through transmembrane transport proteins in the plasma membrane (3-5). The paracellular pathway is associated with passive movement of water and solutes through the space between adjacent cells. The majority of transmucosal transport is attributed to paracellular permeability and this is specifically associated with tight junction function (6-9). The paracellular route also provides major barrier functions as well as transport functions (10). The tight junctions are a multifunctional complex that forms intestinal paracellular barriers. It excludes the transport of toxic macromolecules and microorganisms but allows permeation of ions. Although movement of ions through the tight junction is a passive process that largely depends on the concentration gradients of permeant ions, tight junctions selectively regulate the movement of ions across the epithelium.

The permeability of tight junctions depends on the expression of integral membrane proteins, including occludin and the claudins, as well as the anatomical arrangement of anastomosing strands formed by these proteins. Some organs have “tight” high resistance tight junctions; others have “leaky” low resistance tight junctions (9). For example, small intestinal epithelial cells have “leaky” tight junctions, allowing for exchange of water and nutrients. In contrast, gallbladder epithelial cells have “tight” high resistance tight junctions that obstruct the entrance of bile acids into the blood circulation (11). This permeability of tight junctions is determined by the number of strands and composition of tight junction proteins. (12) Permeability of tight junction barrier also varies along the villus-crypt axis. Freeze fracture electron microscopic techniques have revealed that there are more tight

junction strands between villous epithelium than the epithelium in the crypts. (13) Claudins, tight junction membrane proteins, displayed selective expression along the crypt-villus axis. (14) In conclusion, tight junctions are structural features that help to maintain paracellular permeability for preventing the entry of luminal contents of the gut.

2. Measurement of Tight Junction Selective Permeability

To study mechanisms of epithelial barrier function, it is necessary to sensitively and dynamically measure tight junction permeability. Tight junction permeability is generally measured by transepithelial electrical resistance (TER), dilution potential, bi-ionic substitution, or paracellular flux assay. Since TER measurements reflect the degree to which ions transverse tissue, it is commonly used to assess the integrity of tight junctions. Electrical resistance across a monolayer represents the paracellular resistance. (15). The dilution potential technique is particularly useful for measuring charge selectivity (i.e., the passage of anions versus cations). To induce a transepithelial electrochemical gradient, the apical or basolateral bathing solution is replaced with a solution containing altered Na^+ and Cl^- concentrations. Following a change in ion concentration, the dilution potential stabilizes by establishing a new equilibrium potential based on the relative paracellular permeabilities of Na^+ and Cl^- (16, 17). The Bi-ionic potential approach replaces Na^+ on apical or basolateral sides of the epithelial monolayer or tissue being studied with organic cations of various sizes to determine the size selectivity of the tight junctions (18).

Paracellular probe flux assays are also used to assess the integrity of tight junctions using hydrophilic and uncharged molecules that selectively cross the paracellular space.

Examples of such probes are radioactive or fluorescently conjugated mannitol or dextran (19, 20). Performing these flux assays can be used to calculate unidirectional permeability coefficients to various sizes and shapes of solutes. These approaches provide information about selective permeabilities of tight junctions in *in vitro* monolayer and *in vivo* mucosa.

3. Molecular structure and components of the Tight Junction.

The anatomic structure of the tight junction was initially determined by electron microscopic visualization, with identification of regions where the outer leaflets of plasma membranes from adjacent cells appeared to fuse together and obliterate the intercellular space (20). In ultrathin electron microscopy, tight junctions appear as a series of distinct contacts between the outer leaflets of the membrane of adjacent cells (21). Freeze-fracture microscopy reveals the tight junction as intramembranous networks of anastomosing strands arranged within the apical region of the lateral membrane of epithelial cells. Several studies show that these strands consist of multiple protein complexes of transmembrane, cytoskeletal, and signaling proteins (22). At least three different types of transmembrane proteins have been identified at tight junctions: occludin, claudins and junctional adhesion molecules (JAM) (23). The scaffold PDZ-expressing protein Zonula Occludens proteins (ZOs), peripheral membrane proteins, are also present within the tight junctions (24).

3.1. Occludin

Occludin is a ~60-kDa transmembrane protein that was identified in chicken liver as the first component of tight junction intermembrane strands (25). Hydrophobicity plots of

occludin predict that it contains four transmembrane domains with cytoplasmic N- and C-terminal regions and forms two extracellular loops (Figure 1). Among these domains, the first extracellular loop contains a high content of tyrosine (15). Glycine residues are also highly conserved as are the C-terminal 150 amino acids and play a role in cell to cell coupling (26).

Occludin is highly expressed at tight junctions and seems to be involved in the barrier and fence functions. There is substantial evidence supporting a functional role for occludin. First, the overexpression of occludin in cultured MDCK cells increases the number of tight junction strands and elevates the TER (27, 28). Second, the paracellular leakage of small molecules increases in MDCK cells or Xenopus embryo cells in which C-terminally truncated occludin are expressed (27, 29). Third, when occludin is transfected into occludin-null fibroblasts, occludin increases intercellular adhesion (30). Lastly, occludin-induced adhesion is interrupted by the addition into the culture medium of a synthetic peptide corresponding to second extracellular loops resulting in a decreased TER (31).

However, there is also evidence that occludin is not an essential component to the integrity of tight junctions. For example, occludin-deficient embryonic stem cells aggregated to form embryoid bodies with an outermost layer of epithelial cells similar to wild-type embryonic stem cells (32). In addition, occludin homozygous null mice display intact morphology of tight junctions and barrier function despite post-natal growth retardation and infertility in the male mice (33).

3.2. Claudins

Claudins are a large family of transmembrane proteins that are a component of the tight

junction intercellular strands. To date, 24 members of this family have been identified. All claudins are 20-27 kDa proteins with four transmembrane domains, two extracellular loops, and cytoplasmic N- and C- termini (Figure 1). Tsukita et al. first identified two 22-kDa proteins from occludin-containing chicken liver junctional fractions: Claudin-1 and -2 (34, 35). Claudin family members appear to be expressed in a tissue-specific manner and most cells express anywhere from two to ten claudin isoforms. For example, claudin-1, claudin-5, and claudin-15 are expressed primarily by endothelial cells, claudin-11 is selectively expressed in oligodendrocytes and Sertoli cells (36). Freeze-fracture electron microscopy has revealed that the claudins as well as occludin constitute the tight junction strands (34, 35). Cell-type-specific barrier properties in tight junctions appear to be determined by combination and mixing ratios of multiple claudin family members (37).

Multiple claudin family members have two different functional subcategories in paracellular permeability. Some claudins, called "sealing claudins", decrease paracellular permeability, while others, called "pore-forming claudins", enhance paracellular permeability in a charge-selective fashion (30, 38). The "sealing claudins" include claudins-1, -3, -5, -11, and -9. Claudin-1 is crucial for barrier function as was shown in claudin-1 null mice skin. These mice die within hours after birth because of dehydration induced by an impaired epidermal barrier (39). The "pore-forming claudins" are claudin-2, -7, -15, and -16. Claudin-2 forms a paracellular channel which is selective for small cations, particularly Na^+. Overexpression of claudin-2 in MDCK cells results in a decrease in TER and enhances the selective permeability of small cations (40, 41).

3.3. Junctional adhesion molecules

JAMs are 36-41 kDa single pass transmembrane proteins and are expressed within tight junctions in epithelial and endothelial cells (Figure 1). JAMs are members of the immunoglobulin gene superfamily and are characterized by two extracellular V-type immunoglobulin loops and a cytoplasmic tail (42). JAM-1 localizes to the cell-cell contact site formed by homophilic interactions between the first V-type Ig loops (43). JAM-2 and -3, on the other hand, form heterophilic interactions (44). JAMs play a role in epithelial barrier function, as demonstrated by studies in which JAM antibodies resulted in a decrease in TER recovery and defects in tight junction assembly (22). These results indicate that JAMs regulate tight junction formation.

3.4. Zonula occludens proteins

ZOs are tight junction proteins that are members of the membrane-associated guanylate kinase (MAGUK) homologue family containing three PDZ domains, an SH3 domain, and a non-catalytic guanylate kinase (GuK) homology domain (23). Three isoforms of ZOs have been identified: ZO-1 (210-225 kDa), ZO-2 (180 kDa), and ZO-3 (130 kDa). ZOs are peripheral membrane proteins and are specifically enriched at the points of tight junction membrane contact in polarized epithelial and endothelial cells (45). ZO-1 plays a crucial role in the formation and function of tight junctions. In a calcium-switch assay using epithelial cells derived from ZO-1 knockout mice, the results show that ZO-1 associates initially with adherens junction components prior to final localization at the tight junction

(46). ZO-2 and -3 were originally identified as tight junction proteins due to their co-immunoprecipitation with ZO-1 (47).

4. Protein interactions in the tight junction assembly.

During the formation of tight junctions, the ZO-1/ZO-2/ZO-3 complex interacts with the transmembrane proteins occludin and claudins as well as the cytoplasmic proteins actin, AF-6, the kinase ZAK (Figure 1) (10). ZO proteins bind to the tight junction transmembrane proteins (occludin, claudins, JAM, etc.) and F-actin with their N- and C-termini, respectively (48). The three PDZ domains within the amino-terminal portion of the ZO proteins allow for specialized protein interactions. ZO-1 and ZO-2 were the first junctional components which were identified as having direct interaction with occludin. (45, 49) ZO-3 was also shown to directly bind to the cytoplasmic domain of occludin. (48) The C-terminus of most claudins also interact directly with the PDZ-1 domains of ZO-1, ZO-2, and ZO-3 (50, 51). This interaction of the tight junction proteins appears to link tight junctions to a perijunctional actomyosin ring, which supports and regulates tight junction permeability (52). Until recently, the tight junction was considered to be a stable structure with heavily cross-linked fibrils (53). However, using the technique of fluorescence recovery after photobleaching (FRAP), recent studies have shown that the structure of the tight junction is more dynamic than previously thought. The FRAP assay showed that fluorescent-tagged occludin, claudins, and ZO-1 are highly mobile (9, 54).

5. Regulation of barrier function by cytokines.

Tight junctions and barrier function are regulated by growth factors, cytokines, drugs,

and hormones. Above all, cytokines are particularly crucial mediators of changes in paracellular permeability and disruption of ZO-1 and occludin because of their role in pathologic conditions such as IBD (20).

5.1. TNF-α

The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) is a critical mediator of barrier loss in IBD pathogenesis. TNF-α-induced barrier loss is associated with increases in permeability (55) and with endocytosis of tight junction proteins (9). TNF-α-induced occludin endocytosis requires MLCK, dynamin II, caveolin-1, and cholesterol-enriched, raft-like membrane domains. This was demonstrated using an MLCK inhibitor resulting in decreased TNF-α-induced internalization of occludin in a mouse model (56). Taken together, these results showed that TNF-α- induced MLCK activation triggers caveolin-1 dependent endocytosis of occludin to effect structural and functional tight junction regulation.

5.2. Interleukins

Interleukins (IL) are a large family of cytokines, and several have been studied for effects on paracellular permeability. The IL family includes IL-1, 2, 4, 6, 10, and 13 and variably affect paracellular permeability (55). For example, treatment of the human intestinal epithelial cells line Caco-2 with IL-1 results in reduced expression of occludin protein and enhanced tight junction permeability by activation of the NF-κB pathway (57). IL-13 also increases paracellular permeability to small, uncharged molecules and cations. This IL-13-mediated barrier dysfunction is correlated with increased claudin-2 expression and not

MLCK activation or endocytosis, or reduced occludin expression (58). Increases in claudin-2 expression enhance paracellular permeability by the formation of a selective paracellular channel (35).

5.3. IFN-γ

Interferon-γ (IFN-γ) is a 20-25 kDa glycosylated protein and Th1 pro-inflammatory cytokine found in elevated levels in the intestinal mucosa of IBD patients. During the inflammatory response, IFN-γ modulates epithelial and endothelial cell barrier function as well as immunoregulatory function (59). In the intestinal cell lines HT-29 and T84, direct treatment with IFN-γ increased the paracellular permeability as indicated by a progressive decrease in TER and increase in mannitol and Na^+ fluxes (60). IFN-γ disrupts barrier function by internalization of tight junction proteins ZO-1 and occludin by inducing rearrangement of the actin cytoskeleton (61). Recent studies indicated that IFN-γ increases the expression of Rho associated kinase (ROCK) which induces phosphorylation and activation of the myosin light chain (MLC) (62). Under inflammatory conditions, IFN-γ and TNF-α levels are simultaneously increased. Combined treatment with TNF-α and IFN-γ induced localization into the cytoplasm of tight junction proteins (55).

CONCLUSIONS

Tight junctions play a crucial role in maintaining epithelial barrier function in health and disease conditions. They form the major paracellular barrier to the flux of ions and molecules. Changes in protein interaction within the tight junction, which may be induced by cytokines, is associated with progression of barrier dysfunction and subsequent inflammatory disease. The interactions and regulations of tight junction proteins in intestinal mucosal

barrier function remains incompletely understood. We therefore need to continue to study how interactions of junctional proteins leads to modulation of junctional barrier properties. Although tremendous progress has been made by researchers in the field, many intriguing questions regarding tight junction function remain to be answered.

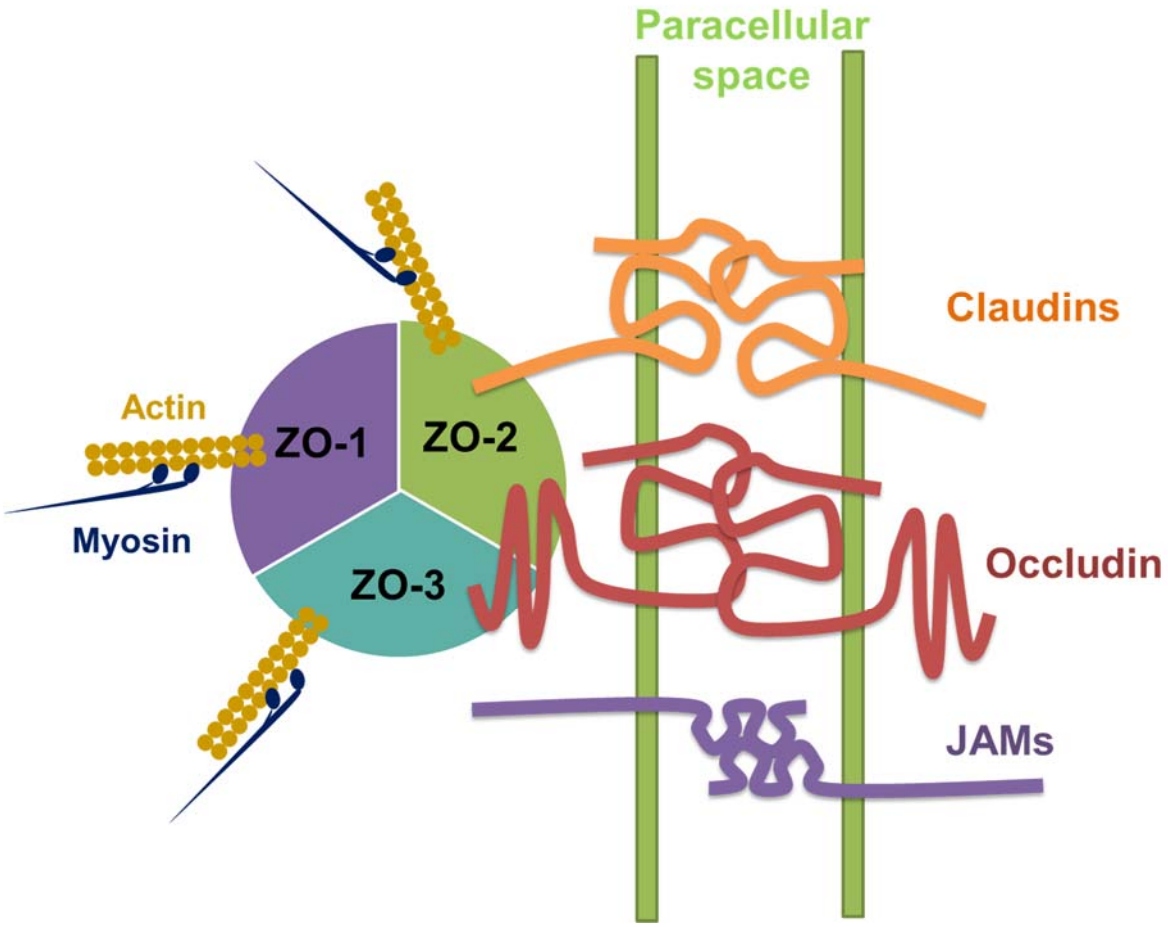


Figure 1. Schematic figure shows protein interactions at the tight junction. ZO-1/ZO-2/ZO-3 complex interact with the claudins and occludin as well as actin-myosin complex.

REFERENCES

1. Farquhar MG, Palade GE. 1963. Junctional Complexes in Various Epithelia. *Journal of Cell Biology* 17: 375-&
2. Mandel LJ, Bacallao R, Zampighi G. 1993. Uncoupling of the molecular 'fence' and paracellular 'gate' functions in epithelial tight junctions. *Nature* 361: 552-5
3. Kunzelmann K, Mall M. 2002. Electrolyte transport in the mammalian colon: Mechanisms and implications for disease. *Physiological Reviews* 82: 245-89
4. Ferraris RP, Diamond J. 1997. Regulation of intestinal sugar transport. *Physiological Reviews* 77: 257-302
5. Broer S. 2008. Amino acid transport across mammalian intestinal and renal epithelia. *Physiological Reviews* 88: 249-86
6. Goodenou.Da, Revel JP. 1970. A Fine Structural Analysis of Intercellular Junctions in Mouse Liver. *Journal of Cell Biology* 45: 272-&
7. Machen TE, Wooding FBP, Erlij D. 1972. Permeable Junctional Complexes - Movement of Lanthanum across Rabbit Gallbladder and Intestine. *Journal of Cell Biology* 54: 302-&
8. Diamond JM, Bossert WH. 1967. Standing-Gradient Osmotic Flow - a Mechanism for Coupling of Water and Solute Transport in Epithelia. *Journal of General Physiology* 50: 2061-&
9. Shen L, Weber CR, Raleigh DR, Yu D, Turner JR. 2011. Tight junction pore and leak pathways: a dynamic duo. *Annu Rev Physiol* 73: 283-309

10. Mitic LL, Van Itallie CM, Anderson JM. 2000. Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. *Am J Physiol Gastrointest Liver Physiol* 279: G250-4

11. Yu D, Turner JR. 2008. Stimulus-induced reorganization of tight junction structure: The role of membrane traffic. *Biochimica Et Biophysica Acta-Biomembranes* 1778: 709-16

12. Anderson JM, Van Itallie CM. 2009. Physiology and function of the tight junction. *Cold Spring Harb Perspect Biol* 1: a002584

13. Lundgren O. 2004. Interface between the intestinal environment and the nervous system. *Gut* 53: 16-8

14. Gunzel D, Yu AS. 2013. Claudins and the modulation of tight junction permeability. *Physiol Rev* 93: 525-69

15. Claude P. 1978. Morphological Factors Influencing Trans-Epithelial Permeability - Model for Resistance of Zonula-Occludens. *Journal of Membrane Biology* 39: 219-32

16. Buchert M, Turksen K, Hollande F. 2012. Methods to examine tight junction physiology in cancer stem cells: TEER, paracellular permeability, and dilution potential measurements. *Stem Cell Rev* 8: 1030-4

17. Barry PH. 2006. The reliability of relative anion-cation permeabilities deduced from reversal (dilution) potential measurements in ion channel studies. *Cell Biochem Biophys* 46: 143-54

18. Kovbasnjuk O, Chatton JY, Friauf WS, Spring KR. 1995. Determination of the Na permeability of the tight junctions of MDCK cells by fluorescence microscopy. *J Membr Biol* 148: 223-32

19. Blikslager AT, Moeser AJ, Gookin JL, Jones SL, Odle J. 2007. Restoration of barrier function in injured intestinal mucosa. *Physiological Reviews* 87: 545-64

20. Harhaj NS, Antonetti DA. 2004. Regulation of tight junctions and loss of barrier function in pathophysiology. *Int J Biochem Cell Biol* 36: 1206-37

21. Stevenson BR, Keon BH. 1998. The tight junction: morphology to molecules. *Annu Rev Cell Dev Biol* 14: 89-109

22. Shin K, Fogg VC, Margolis B. 2006. Tight junctions and cell polarity. *Annu Rev Cell Dev Biol* 22: 207-35

23. Ebnet K. 2008. Organization of multiprotein complexes at cell-cell junctions. *Histochem Cell Biol* 130: 1-20

24. Kojima T, Murata M, Yamamoto T, Lan M, Imamura M, Son S, Takano K, Yamaguchi H, Ito T, Tanaka S, Chiba H, Hirata K, Sawada N. 2009. Tight junction proteins and signal transduction pathways in hepatocytes. *Histol Histopathol* 24: 1463-72

25. Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. 1993. Occludin - a Novel Integral Membrane-Protein Localizing at Tight Junctions. *Journal of Cell Biology* 123: 1777-88

26. AndoAkatsuka Y, Saitou M, Hirase T, Kishi M, Sakakibara A, Itoh M, Yonemura S, Furuse M, Tsukita S. 1996. Interspecies diversity of the occludin sequence: cDNA

cloning of human, mouse, dog, and rat-kangaroo homologues. *Journal of Cell Biology* 133: 43-7

27. Balda MS, Whitney JA, Flores C, Gonzalez 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. *Molecular Biology of the Cell* 7: 3534-

28. McCarthy KM, Skare IB, Stankewich MC, Furuse M, Tsukita S, Rogers RA, Lynch RD, Schneeberger EE. 1996. Occludin is a functional component of the tight junction. *Journal of Cell Science* 109: 2287-98

29. Chen YH, Merzdorf C, Paul DL, Goodenough DA. 1997. C-terminus of occludin is required for tight junction barrier function in early Xenopus embryos. *Molecular Biology of the Cell* 8: 1182-

30. VanItallie CM, Anderson JM. 1997. Occludin confers adhesiveness when expressed in fibroblasts. *Journal of Cell Science* 110: 1113-21

31. Wong V, Gumbiner BM. 1997. A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J Cell Biol* 136: 399-409

32. Saitou M, Fujimoto K, Doi Y, Itoh M, Fujimoto T, Furuse M, Takano H, Noda T, Tsukita S. 1998. Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. *Journal of Cell Biology* 141: 397-408

33. Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, Noda T, Tsukita S. 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Molecular Biology of the Cell* 11: 4131-42

34. Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. 1998. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol* 141: 1539-50

35. Furuse M, Furuse K, Sasaki H, Tsukita S. 2001. Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. *J Cell Biol* 153: 263-72

36. Morita K, Sasaki H, Fujimoto K, Furuse M, Tsukita S. 1999. Claudin-11/OSP-based tight junctions of myelin sheaths in brain and Sertoli cells in testis. *J Cell Biol* 145: 579-88

37. Furuse M, Sasaki H, Tsukita S. 1999. Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol* 147: 891-903

38. Overgaard CE, Daugherty BL, Mitchell LA, Koval M. 2011. Claudins: control of barrier function and regulation in response to oxidant stress. *Antioxid Redox Signal* 15: 1179-93

39. Weng XH, Beyenbach KW, Quaroni A. 2005. Cultured monolayers of the dog jejunum with the structural and functional properties resembling the normal epithelium. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 288: G705-G17

40. Furuse M, Furuse K, Sasaki H, Tsukita S. 2001. Conversion of Zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. *Journal of Cell Biology* 153: 263-72

41. Furuse M, Tsukita S. 2006. Claudins in occluding junctions of humans and flies. *Trends Cell Biol* 16: 181-8

42. Martin-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Panzeri C, Stoppacciaro A, Ruco L, Villa A, Simmons D, Dejana E. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol* 142: 117-27

43. Bazzoni G, Martinez-Estrada OM, Mueller F, Nelboeck P, Schmid G, Bartfai T, Dejana E, Brockhaus M. 2000. Homophilic interaction of junctional adhesion molecule. *J Biol Chem* 275: 30970-6

44. Cunningham SA, Rodriguez JM, Arrate MP, Tran TM, Brock TA. 2002. JAM2 interacts with alpha4beta1. Facilitation by JAM3. *J Biol Chem* 277: 27589-92

45. Itoh M, Morita K, Tsukita S. 1999. Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and alpha catenin. *J Biol Chem* 274: 5981-6

46. Umeda K, Matsui T, Nakayama M, Furuse K, Sasaki H, Furuse M, Tsukita S. 2004. Establishment and characterization of cultured epithelial cells lacking expression of ZO-1. *J Biol Chem* 279: 44785-94

47. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. 2003. Tight junction proteins. *Prog Biophys Mol Biol* 81: 1-44

48. Haskins J, Gu L, Wittchen ES, Hibbard J, Stevenson BR. 1998. ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. *J Cell Biol* 141: 199-208

49. Furuse M, Itoh M, Hirase T, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. 1994. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J Cell Biol* 127: 1617-26

50. Itoh M, Furuse M, Morita K, Kubota K, Saitou M, Tsukita S. 1999. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J Cell Biol* 147: 1351-63

51. Hossain Z, Hirata T. 2008. Molecular mechanism of intestinal permeability: interaction at tight junctions. *Mol Biosyst* 4: 1181-5

52. Cordenonsi M, D'Atri F, Hammar E, Parry DA, Kendrick-Jones J, Shore D, Citi S. 1999. Cingulin contains globular and coiled-coil domains and interacts with ZO-1, ZO-2, ZO-3, and myosin. *J Cell Biol* 147: 1569-82

53. Marchiando AM, Graham WV, Turner JR. 2010. Epithelial barriers in homeostasis and disease. *Annu Rev Pathol* 5: 119-44

54. Johnson LG. 2005. Applications of imaging techniques to studies of epithelial tight junctions. *Adv Drug Deliv Rev* 57: 111-21

55. Capaldo CT, Nusrat A. 2009. Cytokine regulation of tight junctions. *Biochim Biophys Acta* 1788: 864-71

56. Marchiando AM, Shen L, Graham WV, Weber CR, Schwarz BT, Austin JR, 2nd, Raleigh DR, Guan Y, Watson AJ, Montrose MH, Turner JR. 2010. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. *J Cell Biol* 189: 111-26

57. Al-Sadi RM, Ma TY. 2007. IL-1beta causes an increase in intestinal epithelial tight junction permeability. *J Immunol* 178: 4641-9

58. Weber CR, Raleigh DR, Su L, Shen L, Sullivan EA, Wang Y, Turner JR. 2010. Epithelial myosin light chain kinase activation induces mucosal interleukin-13 expression to alter tight junction ion selectivity. *J Biol Chem* 285: 12037-46

59. Walsh SV, Hopkins AM, Nusrat A. 2000. Modulation of tight junction structure and function by cytokines. *Adv Drug Deliv Rev* 41: 303-13

60. Heyman M, Desjeux JF. 2000. Cytokine-induced alteration of the epithelial barrier to food antigens in disease. *Ann N Y Acad Sci* 915: 304-11

61. Chiba H, Kojima T, Osanai M, Sawada N. 2006. The significance of interferon-gamma-triggered internalization of tight-junction proteins in inflammatory bowel disease. *Sci STKE* 2006: pe1

62. Feng J, Ito M, Ichikawa K, Isaka N, Nishikawa M, Hartshorne DJ, Nakano T. 1999. Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J Biol Chem* 274: 37385-90

CHAPTER 2

LITERATURE REVIEW

ROLE OF ClC-2 CHLORIDE CHANNEL IN REGULATION OF INTESTINAL BARRIER FUNCTION

INTRODUCTION

The gastrointestinal epithelial cells form the body's largest interface between biological compartments. These cells allow for the absorption of nutrients while providing a physical barrier to the permeation of proinflammatory molecules, including pathogens, toxins, and antigens, from the luminal environment into the mucosal tissues and circulatory system. The intestinal barrier is composed of a thin lining of mucous and forms a physical barrier that separates the luminal contents from the interstitium. Tight junctions have a crucial role in maintaining the intestinal barrier, and can be altered acutely or long-term by physiological and pathological factors (1-3). Our research group revealed that the ClC-2 chloride channel has a key role in regulating the barrier function in various intestinal diseases (4-8). Our lab also demonstrated that the ClC-2 activator, lubiprostone, has a barrier recovery/protective effect in ischemic injury and experimental colitis models (9-11). However, the function of ClC-2 and mechanisms of action of lubiprostone are controversial. This review summarizes the role of ClC-2 and its activator, lubiprostone, in intestinal barrier function and suggests potential therapeutic targets of ClC-2 in diseases that compromise the intestinal barrier.

1. Defect of Intestinal Mucosal Barrier in Intestinal Disorders

The importance of an intact epithelial tight junction becomes evident in intestinal disorders. The impaired tight junction structural complexity was revealed using electron microscopy in tissues suffering from several intestinal disorders including Crohn's disease (CD) (12), ulcerative colitis (UC) (13) and ischemic injury model (14). Dysregulation of tight junction proteins contributes to barrier loss in patients with intestinal diseases. Claudin-2, a

pore forming tight junction protein, was significantly upregulated in CD (12), UC (15), and collagenous colitis (16) patients by Th2 cell cytokine (IL-13)-dependent mechanism. Occludin and sealing claudins (claudin-1, 3, and 4) were diminished or redistributed in intestinal permeability disorders, including in ischemic injury model (8), CD (12), and UC (17). Reorganization of occludin and sealing claudins was mediated by cytokines (TNFα, IFNγ, LIGHT, and IL-1β) and promoted myosin light chain kinase (MLCK) activation. These pro-inflammatory cytokines promote transcription of MLCK, which when activated, phosphorylates myosin II, inducing endocytosis of tight junction proteins (18-26). However, intestinal mucosal barrier dysfunction can also be caused by epithelial damage regardless of tight junction function, including apoptosis, erosion, and ulceration (27).

2. ClC-2 Chloride Channel

The ClC-0 chloride channel was originally discovered by expression cloning of the *Torpedo marmorata* electric organ (28). To date, nine mammalian CLC family members have been discovered, and they can be divided into three homology groups: ClC-1, -2, -Ka/K1, and –Kb/K2; ClC-3, -4, and -5; and ClC-6 and -7 (29, 30). The ClC-2 chloride channel has 18 helices that partially span the membrane. The two halves of the double barreled structure form two identical, largely independent pores that have a binding site for chloride (31-33). ClC-2 is expressed in the plasma membranes of epithelia cells from many mammalian tissues, including the brain, pancreas, lung, intestine, kidney, liver, and heart (34). Activation of ClC-2 occurs under various physiological conditions including hypo-osmotic shock, hyperpolarization, and acidic extracellular pH and in cellular stress (35-42). The physiological processes of ClC-2 is involved in several mammalian cell types,

including Sertoli cells (43), sympathetic (44) and hippocampal neurons (45, 46), rod bipolar cells (47), hepatocytes (48), erythrocytes (49), trabecular meshwork cells (50), colon epithelial cells (51), pancreatic acinar cells (52), as well as salivary acinar (53) and duct (54) cells. In addition, the testicular and retinal degeneration (43) as well as leukodystrophy (55) observed in $ClC2^{-/-}$ mice suggest a crucial role of ClC-2 chloride channel in the control of ionic environment in the germinal and retinal epithelia as well as central nervous system.

3. Role of ClC-2 chloride channel in intestinal mucosal homeostasis.

Although ClC-2 chloride channels contribute to chloride secretion in cultured intestinal cells and murine and pig intestinal epithelium (4, 56, 57), the physiological contribution of ClC-2 to chloride secretion remains unclear. There is some evidence suggesting that ClC-2 does not contribute to fluid secretion. Foremost, ClC-2 is predominantly located in intestinal villus epithelia rather than in the secretory crypts epithelia (58-60). Secondly, $ClC\text{-}2^{-/-}$ mice do not show any secretory functional change in gastric acid secretion (61) and intestinal chloride secretion (37). Finally, ClC-2-CFTR (cystic fibrosis transmembrane conductance regulator) double-knockout mice do not exhibit more severe pathogenic effects of CFTR disruption in mice (37).

ClC-2 chloride channels are located in proximity to tight junctions on the lateral membrane of the murine villus enterocyte (56, 62) . Furthermore, our previous studies have shown that ClC-2 was located in close proximity to the tight junction area in porcine (4) and murine (8) intestine. However, there is debate concerning the cellular and membrane location of ClC-2. The location of ClC-2 varies depending on species and tissue. Several researchers have shown that ClC-2 is located in the basolateral membrane of the intestinal epithelia near

or at the tight junction region, with the exception of a small number of studies (Table 1) (4, 5, 7, 8, 56, 62-67).

The unusual lateral expression of ClC-2 near the tight junction region presented questions regarding its role in the regulation of tight junctions. Our lab has accumulated evidence regarding the role of ClC-2 in tight junction regulation at the intestinal barrier. We have also examined the role of ClC-2 chloride channels in regulating intestinal barrier function using a ClC-$2^{-/-}$ mice model (6) and ClC-2 knockdown in human intestinal epithelia Caco-2 BBe cells (7). The functional and morphological alterations of the tight junction barrier were observed in the intestinal mucosa of ClC-$2^{-/-}$ mice. Our lab group has shown that the ClC-$2^{-/-}$ mice have reduced baseline paracellular permeability and altered tight junction morphology (Fig. 1) (6). In addition, ClC-2 knockdown human intestinal epithelial Caco-2 cells showed impaired development of barrier function and disrupted localization of occludin via caveolin-1-dependent manner (7). Taken together, these results strongly suggest that ClC-2 has a critical role in the modulation of the tight junction barrier to maintain the intestinal mucosal barrier functions.

4. ClC-2 chloride channel is a key factor in maintaining intestinal barrier during intestinal disorders.

4.1. Ischemia-injured intestine disease model.

We first reported that barrier function recovery in ischemia-injured porcine ileum initiated by chloride secretion via ClC-2 chloride channels co-expressed with occludin. Application of prostaglandin E_2 (PGE_2) to ischemic-injured ileal mucosa stimulated increases

in short-circuit current (Isc, an indicator of Cl^- secretion) that was followed by marked increases in transepithelial resistance (TER, an indicator of barrier function recovery). *In vitro* studies revealed that recovery of barrier functions was initiated by Cl^- secretion via ClC-2 chloride channels co-expressed with occludin and localized to tight junctions within restituting epithelium (4). ClC-$2^{-/-}$ mice had increased paracellular permeability in jejunal mucosa following ischemic injury compared to wild type mice. Electronmicroscopic examination of recovering tissue revealed tight junction dilation in ClC-$2^{-/-}$ mice, whereas wild type epithelium had tightly opposed tight junctions. In a confocal immunofluorescence study, the tight junction protein, occludin, was co-localized with ClC-2 in the tight junction region. The occludin was internalized during post-ischemic recovery, but regained its normal pattern after 3-hours post-ischemic recovery. In ClC-2 deficient mice intestine, however, the occludin remained diffusely present within the subapical region even after 3-hours post-ischemic recovery (Fig. 2) (8). Collectively, these findings indicated that ClC-2 plays a key role in restoration of the intestinal epithelium barrier by anchoring assembly of tight junctions following ischemic injury.

4.2. Inflammatory bowel disease

Defect of intestinal barrier function is known to contribute to inflammatory bowel disease (IBD) progression (12, 13, 15). Thus we hypothesized that the ClC-2 chloride channel also has a critical role in the progression and severity of intestinal inflammatory response by regulating intestinal barrier. Our recent study found that the severity of experimental colitis was significantly higher in the ClC-$2^{-/-}$ mice as compared with WT mice. ClC-$2^{-/-}$ mice had a higher disease activity index, higher histological scores, and increased

paracellular permeability compared with wild-type mice when treated with DSS. Dextran sulfate sodium (DSS)-treated ClC-2 deficient mice had increased claudin-2 (pore-forming claudin) expression, and greater loss of occludin in the apical membrane of colonic mucosa (Fig. 3). Thus, absence of ClC-2 triggers destabilization of tight junction proteins to increase barrier permeability during DSS-induced colitis. ClC-2 knockdown in Caco-2BBe cells resulted in a significant loss of TER in the DSS treatment group compared to wild type cells. The protein and mRNA expression of ClC-2 was dramatically reduced in colonic biopsies from UC patients. Taken together, we concluded that ClC-2 plays a key role in regulation of the tight junction barrier functions in the development of DSS-induced murine colitis (5). ClC-2 will be a molecular target for improvement of therapeutic efforts in intestinal barrier defect diseases including DC, UC, and ischemic injury.

5. Drug targeting ClC-2

5.1. Lubiprostone

Lubiprostone (Amitiza, RU-0211), a ClC-2 activator, is a bicyclic fatty acid compound derived from a metabolite of prostaglandin E1 (PGE_1) (68). Activation of ClC-2 channels by lubiprostone result in efflux of chloride into the lumen of the gastrointestinal tract and promotes intestinal fluid secretion (69, 70). The drug is used as a treatment of chronic idiopathic constipation (CIC) and irritable bowel syndrome (IBS) with constipation (71-73). The mechanism of action of lubiprostone in the intestine originally proposed that it directly activates ClC-2 chloride channels without affecting CFTR on the apical membrane of human colonic T84 cell (63, 74). However, mechanisms of lubiprostone-induced ClC-2-

mediated chloride secretion remain controversial. Several papers recently published suggest that lubiprostone opens the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel via the prostaglandin E receptor 4 (EP4) and cAMP signaling, without affecting ClC-2 (75-77). Other laboratories have extended these studies, and have detected activation of dual activation of CFTR and ClC-2 in a dose dependent manner (74, 78). In addition, there are several mechanisms of action of lubiprostone revealed by recent studies including ion transporter trafficking, mucus release, and smooth muscle contraction (Table 2.) (9, 11, 63, 67, 70, 74-92).

5.2. Lubiprostone in intestinal barrier dysfunction

Previous studies showed that lubiprostone promoted repair of barrier properties in a ClC-2-dependent manner in ischemic injured intestine (9-11). Treatment with lubiprostone to ischemia-injured mucosa induced increased TER and significantly reduced mucosal-to-serosal fluxes of ^{3}H-labeled mannitol. During peak recovery of TER in ischemic tissue, the occludin was localized exclusively to the tight junction area in lubiprostone treated tissues compare to the diffused occludin staining in untreated tissues (9). Lubiprostone also showed the protective and reparative properties in proinflammatory cytokines exposure of T84 cell. Using measurements of TER, fluxes of fluorescent macromolecules, occludin and mitochondrial membrane potential, lubiprostone protected or promoted repair of epithelial barrier and cell function against damage caused by interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). These barrier protective/reparative properties were diminished by a ClC-2 inhibitor (methadone) indicating that the barrier protective effect of lubiprostone is dependent on ClC-2 (11). In our laboratory's unpublished data, lubiprostone has a protective

or therapeutic effect on the DSS- and TNBS-induced murine colitis model and also showed barrier protective effects. Treatment with lubiprostone reduced body weight loss, disease activity index, colon shortening, histological score, and intestinal permeability. Oral treatment of lubiprostone in DSS-induced colitis also induced reconstitutions of tight junction proteins. Using immunofluorescence confocal microscopy analysis of the tight junction proteins, lubiprostone treatment recovered dislocalization of ZO-1, occludin, and claudin-1, and reduced expression of claudin-2 in apical membrane of the DSS colitis mice colon. In addition, treatment with lubiprostone in DSS colitis mice showed reduced crypt height and PCNA ratio, indicators of epithelial proliferation and undifferentiated cells, and elevated goblet cell numbers. However, this drug showed very limited protective effect in the ClC-$2^{-/-}$ mice DSS colitis model. Oral treatment with lubiprostone in ClC-$2^{-/-}$ mice DSS colitis didn't show significant change in tight junction, crypts height, and PCNA ratio, but increased goblet cell numbers. Taken together, these results indicated that the protective effect of the lubiprostone caused by reconstitution of tight junction proteins and epithelial differentiation in a ClC-2 dependent manner. In conclusion, lubiprostone has a major protective effect, which is dependent upon ClC-2 chloride channel in experimental murine models of colitis. However, it also has some alternative protective mechanisms of action including goblet cell proliferation. Additional investigation is required in order to determine the detailed mechanisms of action of lubiprostone in diseases of intestinal barrier dysfunction. This may be beneficial to patients suffering from impaired epithelial barrier function, which occurs in IBD and IBS.

CONCLUSION

The intestinal tight junction barrier is dynamically regulated by physiological and pathological factors, including growth factors, cytokines, drugs, hormones, and ion channels(1-3). However, the mechanisms of interaction and regulation of tight junction proteins remains elusive. We found that the ClC-2 chloride channel has a critical role in regulation of tight junctions and barrier function, and we also showed that the ClC-2 activator, lubiprostone, has a barrier recovery/protective effect in murine models of intestinal dysfunction diseases. Still, we still have questions about that how ClC-2 regulates the tight junction barrier as well as how to develop a ClC-2 specific activator to minimize unexpected side effects. Thus, we need to study understanding the molecular mechanisms underlying tight junction regulation by the ClC-2 chloride channel. This will lead to the development of effective therapeutic and preventive targets promising to help treat intestinal diseases associated with intestinal barrier dysfunctions.

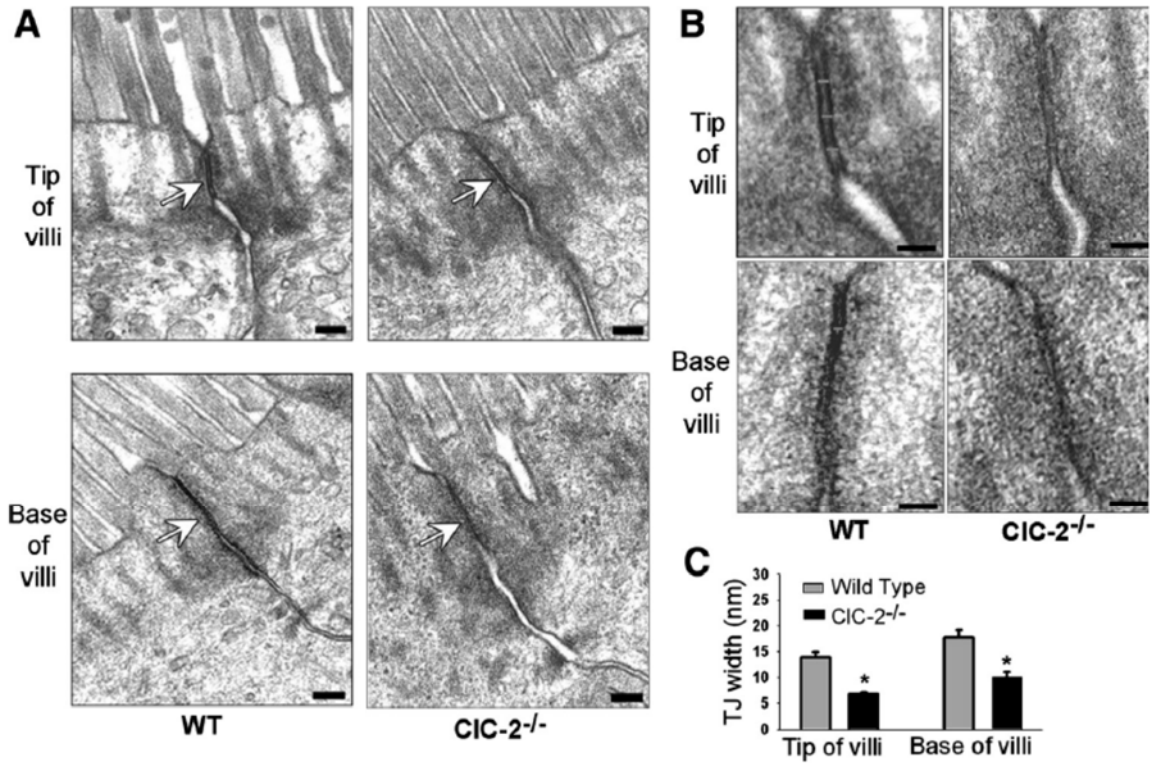


Figure 1. Tight junction ultrastructure in WT and ClC-2$^{-/-}$ mouse small intestine. A: in WT intestine, apical intercellular tight junctions appeared to be more electron dense and closely apposed compared with ClC-2$^{-/-}$intestine, in which the tight junctions revealed lateral membranes that were less well defined but closely aligned (arrows). Bars, 200 nm. B: higher magnification of respective images in A. Gray lines indicate measurements of tight junction width. Bars, 50 nm. C: width of apical tight junctions (TJ) was reduced by ~50% in ClC-2$^{-/-}$ intestine. *P<0.01. [Image adapted from, reference (94), with permission.]

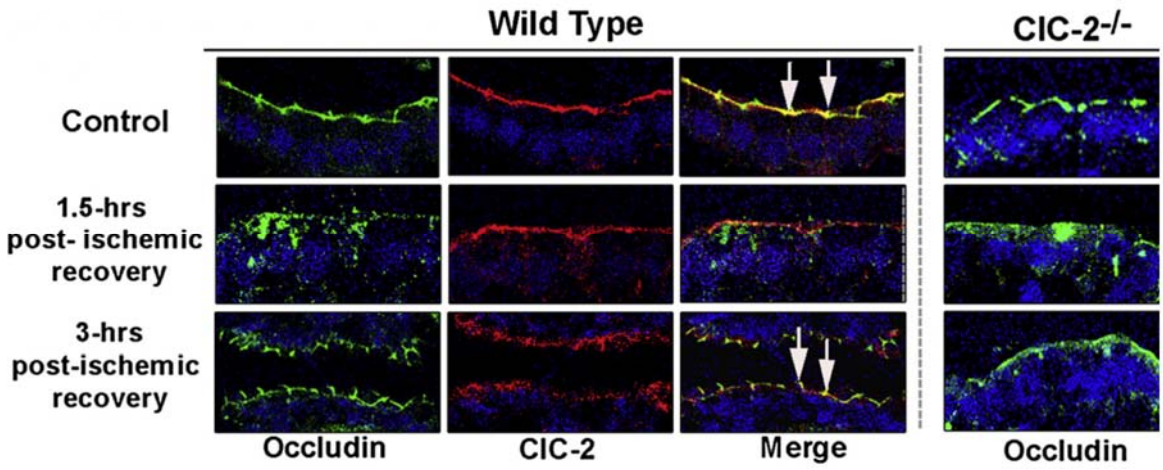


Figure 2. ClC-2 and occludin co-localization using confocal immunofluorescence. In wild type and ClC-$2^{-/-}$ mice control intestine, occludin (green) was localized to apical tight junctions (first row). In wild type control intestine ClC-2 (red) was localized along the apical border, concentrated around the tight junction region, and co-localized with occludin (yellow color in the merged figure).During post-ischemic recovery, occludin fluorescence was diffuse, but regained its normal distribution after 3 h of recovery in wild type mice (middle and lower row). However in ClC-$2^{-/-}$ mice, occludin localization was diffuse even after 3 h of recovery (far right panel). Nuclei (blue) were stained with DRAQ5 (Biostatus Ltd.). All images were viewed with a 60× objective and 1.5× zoom. [Image adapted from, reference (39), with permission.]

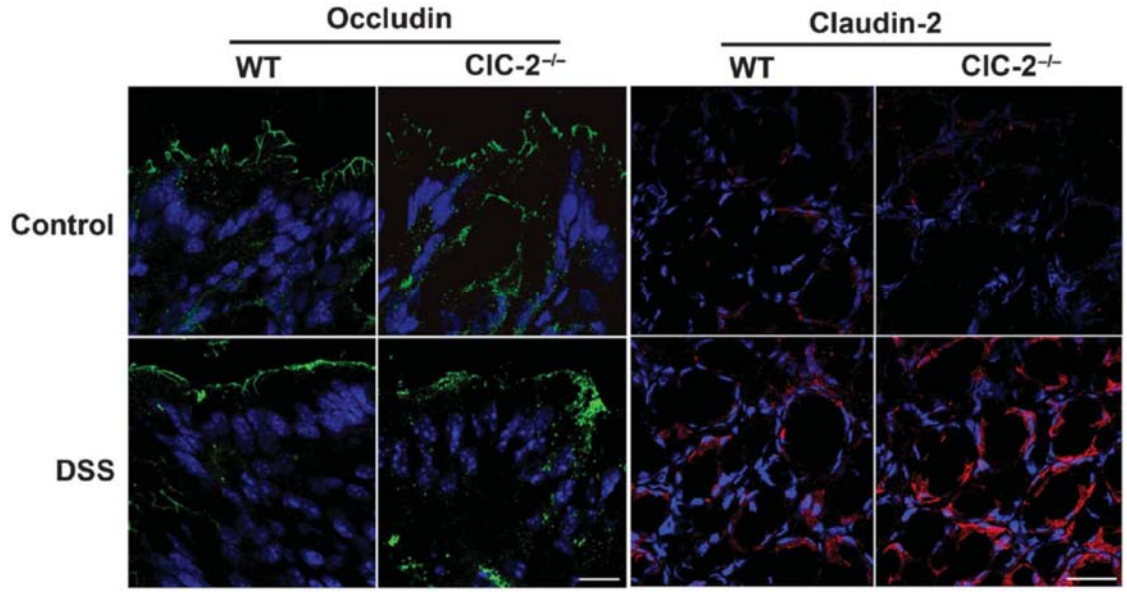


Figure 3. Tight junction analysis. In confocal immunofluorescence, the occludin (green) staining on surface epithelium was diffused and subapically distributed in the colon of ClC-$2^{-/-}$ DSS mice compared with the colon of WT DSS mice. The staining of claudin-2 (red) was found to be increased in colonic crypts after DSS administration. The DSS-induced increase in the intensity of claudin-2 staining was higher in ClC-$2^{-/-}$ DSS mice compared with WT DSS mice. White bar = 50 μM. [Image adapted from, reference (92), with permission.]

Table 1. Distribution of ClC-2 chloride channel in various species and tissues.

Research Groups	Species	Cells/Tissues	Api	BL	TJ	Cyt	Refferences
Cuppoletti, J.	Human	T84	+				Cuppoletti et al., 2004.
Melvin, J.E.	Mice	Early distal colon		+		+	Catalan et al., 2012
		Late distal colon		+		+	
Sepulveda, F.V.	Human	Caco-2		+			Pena-Munzenmayer et al., 20
	Mice	Duodenum		+			
Bear, C.E.	Human	Caco-2	+		+		Mohammad-Panah et al., 200
	Mice	Small intestine			+		Gyomorey et al., 2000
Fritsch, J.	Rat	Small intestine		+			Lipecka et al., 2002
		Colon enterocytes		+			
	Human	Colon enterocytes				+	
Blikslager, A.T.	Pig	ileum	+		+		Moeser et al., 2004
	Mice	Jejunum	+		+		Nighot et al., 2009
	Human	Caco-2	+			+	Nighot et al., 2012
	Mice	Colon	+			+	Nighot et al., 2013
	Human	Colon				+	
Ammen, N.A.	Rat	Proximal colon		+			Jakab et al., 2012
	Human	Colon		+			

Api: apical membrane, BL: basolateral membrane, TJ: tight junction, Cyt: cytosol

Table 2. Mechanisms of actions of lubiprostone.

Mechanisms of action	Targets of action	Signalings	Models	Results	References
Cl^- secretion	ClC-2	independent of PKA	T84 cells ClC-2 expressing HEK-293 cells	↑ Cl^- currents (I_{sc})	Cuppoletti et al., 2004
		-	CFTR KD or WT Calu-3 cells	↑ Cl^- currents (I_{sc})	MacVinish et al., 2007
		more selective than PGE_2	ischemic porcine ileum	↑ Cl^- currents (I_{sc}) ↑ Barrier (TER/Flux)	Moeser et al., 2007
		-	nasal epithelium in CF mice	↑ Cl^- secretion (NPD)	MacDonald et al., 2008
		-	ischemic porcine jejunum Cytokines-induced damaged T84 cells	↑ Cl^- currents (I_{sc}) ↑ Barrier (TER/Flux)	Cuppoletti et al., 2012
		independent of enteric nerve independent of PGs	Guinea pig small intestine and colon	↑ Cl^- currents (I_{sc})	Fei et al., 2009
	ClC-2 (low dose) /CFTR (high dose)	-	A6 cell	↑ Cl^- currents (I_{sc}) ↑ Voltage	Boa et al., 2008
	ClC-2/CFTR	-	murine nasal epithelium	↑ Cl^- secretion (NPD)	Schiffhauer et al., 2013
	CFTR	EP_4 receptor/cAMP/PKA	T84 cells Intestinal epithelium of WT and CF mice Intestinal epithelium of CF patient and controls	↑ Cl^- currents (I_{sc})	Bijvelds et al., 2009
		cAMP/PKA	T84 cells	↑ Cl^- currents (I_{sc})	Ao et al., 2011
		EP_4 receptor	*Xenopus* oocytes	↑ anion conductance	Norimatsu et al., 2012
		EP_4 receptor/cAMP	ventral tracheal epithelium of sheep	↑ Cl^- currents (I_{sc})	Cuthbert, 2011
HCO_3^- secretion	CFTR	EP_4 receptor	rat proximal duodenal loops	↑ HCO_3^- secretion	Mizumori et al., 2009
smooth muscle contraction	EP_1 receptor EP_4 receptor	-	rat and human stomach longitudinal mucsle rat and human colon circular mucsle	↑ contraction ↓ neuronally mediated contraction	Bassil et al., 2008
	independent of EP receptor	-	human uterine smooth muscle cells	↓ $[Ca^{2+}]$ ↑ membrane potential	Cuppoletti et al., 2008
	EP_1 receptor	-	murine small intestine (longitudinal) murine small intestine (circular) murine pyloric tissue	No effect on contraction ↑ EFS-induced contraction ↑ basal tone	Chan and Mahimo, 2013
	independent of CFTR	-	mice small intestine	↑ small intestine transit	De Lisle et al., 2010
Mucosal contractility	Serotonergic, EP4/PKA and EP1	-	human and rat colon	↑ contraction of villi proximal colonic *plicae*	Jakab et al., 2012
Mucus seretion	ClC-2, CaCC, and CFTR	-	human airway epithelia	↑ gland secretion	Joo et al., 2009
	independent of CFTR	-	CF mice	↑ mucus accumulation in the crypts	De Lisle et al., 2010
	EP_4 receptor ?	-	mouse proximal-mid small intestine	↑ mucin release	De Lisle, 2012
	independent of CFTR	-	mouse proximal and distal colon	↓ inner mucus layer thickness	Musch et al., 2013

PKA: Protein kinase A, PG: Prostaglandin, EP: prostaglandin E, CaCC: Calcium-activated chloride channel, CF: cystic fibrosis, EFS: electrical field stimulation, Isc: short-circuit current, TER: Transepithelial Resistance, Flux: mucosal-to-serosal fluxes of 3H-labeled mannitol, NPD: Narsal potential difference

REFERENCES

1. Harhaj NS, Antonetti DA. 2004. Regulation of tight junctions and loss of barrier function in pathophysiology. *International Journal of Biochemistry & Cell Biology* 36: 1206-37
2. Suzuki T. 2013. Regulation of intestinal epithelial permeability by tight junctions. *Cellular and Molecular Life Sciences* 70: 631-59
3. Rajasekaran SA, Beyenbach KW, Rajasekaran AK. 2008. Interactions of tight junctions with membrane channels and transporters. *Biochim Biophys Acta* 1778: 757-69
4. Moeser AJ, Haskell MM, Shifflett DE, Little D, Schultz BD, Blikslager AT. 2004. ClC-2 chloride secretion mediates prostaglandin-induced recovery of barrier function in ischemia-injured porcine ileum. *Gastroenterology* 127: 802-15
5. Nighot P, Young K, Nighot M, Rawat M, Sung EJ, Maharshak N, Plevy SE, Ma T, Blikslager A. 2013. Chloride Channel ClC-2 is a Key Factor in the Development of DSS-induced Murine Colitis. *Inflamm Bowel Dis*
6. Nighot PK, Blikslager AT. 2010. ClC-2 regulates mucosal barrier function associated with structural changes to the villus and epithelial tight junction. *Am J Physiol Gastrointest Liver Physiol* 299: G449-56
7. Nighot PK, Blikslager AT. 2012. Chloride channel ClC-2 modulates tight junction barrier function via intracellular trafficking of occludin. *Am J Physiol Cell Physiol* 302: C178-87

8. Nighot PK, Moeser AJ, Ryan KA, Ghashghaei T, Blikslager AT. 2009. ClC-2 is required for rapid restoration of epithelial tight junctions in ischemic-injured murine jejunum. *Exp Cell Res* 315: 110-8

9. Moeser AJ, Nighot PK, Engelke KJ, Ueno R, Blikslager AT. 2007. Recovery of mucosal barrier function in ischemic porcine ileum and colon is stimulated by a novel agonist of the ClC-2 chloride channel, lubiprostone. *Am J Physiol Gastrointest Liver Physiol* 292: G647-56

10. Moeser AJ, Nighot PK, Roerig B, Ueno R, Blikslager AT. 2008. Comparison of the chloride channel activator lubiprostone and the oral laxative Polyethylene Glycol 3350 on mucosal barrier repair in ischemic-injured porcine intestine. *World J Gastroenterol* 14: 6012-7

11. Cuppoletti J, Blikslager AT, Chakrabarti J, Nighot PK, Malinowska DH. 2012. Contrasting effects of linaclotide and lubiprostone on restitution of epithelial cell barrier properties and cellular homeostasis after exposure to cell stressors. *BMC Pharmacol* 12: 3

12. Zeissig S, Burgel N, Gunzel D, Richter J, Mankertz J, Wahnschaffe U, Kroesen AJ, Zeitz M, Fromm M, Schulzke JD. 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

13. Das P, Goswami P, Das TK, Nag T, Sreenivas V, Ahuja V, Panda SK, Gupta SD, Makharia GK. 2012. Comparative tight junction protein expressions in colonic

Crohn's disease, ulcerative colitis, and tuberculosis: a new perspective. *Virchows Archiv* 460: 261-70

14. Little D, Dean RA, Young KM, McKane SA, Martin LD, Jones SL, Blikslager AT. 2003. PI3K signaling is required for prostaglandin-induced mucosal recovery in ischemia-injured porcine ileum. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 284: G46-G56

15. Heller F, Florian P, Bojarski C, Richter J, Christ M, Hillenbrand B, Mankertz J, Gitter AH, Burgel N, Fromm M, Zeitz M, Fuss I, Strober W, Schulzke JD. 2005. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology* 129: 550-64

16. Burgel N, Bojarski C, Mankertz J, Zeitz M, Fromm M, Schulzke JD. 2002. Mechanisms of diarrhea in collagenous colitis. *Gastroenterology* 123: 433-43

17. Schmitz H, Barmeyer C, Fromm M, Runkel N, Foss HD, Bentzel CJ, Riecken EO, Schulzke JD. 1999. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. *Gastroenterology* 116: 301-9

18. Wang FJ, Graham WV, Wang YM, Witkowski ED, Schwarz BT, Turner JR. 2005. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. *American Journal of Pathology* 166: 409-19

19. Graham WV, Wang FJ, Clayburgh DR, Cheng JX, Yoon B, Wang YM, Lin AN, Turner JR. 2006. Tumor necrosis factor-induced long myosin light chain kinase

transcription is regulated by differentiation-dependent signaling events - Characterization of the human long myosin light chain kinase promoter. *Journal of Biological Chemistry* 281: 26205-15

20. Ma TY, Boivin MA, Ye DM, Pedram A, Said HM. 2005. Mechanism of TNF-alpha modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 288: G422-G30

21. Zolotarevsky Y, Hecht G, Koutsouris A, Gonzalez DE, Quan C, Tom J, Mrsny RJ, Turner JR. 2002. A membrane-permeant peptide that inhibits MLC kinase restores barrier function in in vitro models of intestinal disease. (vol 123, pg 163, 2002). *Gastroenterology* 123: 1412-

22. Clayburgh DR, Barrett TA, Tang YM, Meddings JB, Van Eldik LJ, Watterson DM, Clarke LL, Mrsny RJ, Turner JR. 2005. Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo. *Journal of Clinical Investigation* 115: 2702-15

23. Blair SA, Kane SV, Clayburgh DR, Turner JR. 2006. Epithelial myosin light chain kinase expression and activity are upregulated in inflammatory bowel disease. *Laboratory Investigation* 86: 191-201

24. Schwarz BT, Wang FJ, Shen L, Clayburgh DR, Su LP, Wang YM, Fu YX, Turner JR. 2007. LIGHT signals directly to intestinal epithelia to cause barrier dysfunction via cytoskeletal and endocytic mechanisms. *Gastroenterology* 132: 2383-94

25. Al-Sadi R, Ma TY. 2007. Myosin light chain kinase (MLCK) mediates the IL-1 beta-incluced increase in intestinal epithelial Tight Junction permeability. *Gastroenterology* 132: A8-A

26. Al-Sadi RM, Ma TY. 2007. IL-1 beta causes an increase in intestinal epithelial tight junction permeability. *Journal of Immunology* 178: 4641-9

27. Gitter AH, Wullstein F, Fromm M, Schulzke JD. 2001. Epithelial barrier defects in ulcerative colitis: Characterization and quantification by electrophysiological imaging. *Gastroenterology* 121: 1320-8

28. Jentsch TJ, Steinmeyer K, Schwarz G. 1990. Primary structure of Torpedo marmorata chloride channel isolated by expression cloning in Xenopus oocytes. *Nature* 348: 510-4

29. Stauber T, Weinert S, Jentsch TJ. 2012. Cell biology and physiology of CLC chloride channels and transporters. *Compr Physiol* 2: 1701-44

30. Strange K. 2002. Of mice and worms: novel insights into ClC-2 anion channel physiology. *News Physiol Sci* 17: 11-6

31. Dutzler R, Campbell EB, Cadene M, Chait BT, MacKinnon R. 2002. X-ray structure of a ClC chloride channel at 3.0 A reveals the molecular basis of anion selectivity. *Nature* 415: 287-94

32. Ramjeesingh M, Li C, She YM, Bear CE. 2006. Evaluation of the membrane-spanning domain of ClC-2. *Biochem J* 396: 449-60

33. Ramjeesingh M, Li C, Huan LJ, Garami E, Wang Y, Bear CE. 2000. Quaternary structure of the chloride channel ClC-2. *Biochemistry* 39: 13838-47

34. Thiemann A, Grunder S, Pusch M, Jentsch TJ. 1992. A chloride channel widely expressed in epithelial and non-epithelial cells. *Nature* 356: 57-60

35. Jordt SE, Jentsch TJ. 1997. Molecular dissection of gating in the ClC-2 chloride channel. *EMBO J* 16: 1582-92

36. Grunder S, Thiemann A, Pusch M, Jentsch TJ. 1992. Regions involved in the opening of CIC-2 chloride channel by voltage and cell volume. *Nature* 360: 759-62

37. Zdebik AA, Cuffe JE, Bertog M, Korbmacher C, Jentsch TJ. 2004. Additional disruption of the ClC-2 Cl(-) channel does not exacerbate the cystic fibrosis phenotype of cystic fibrosis transmembrane conductance regulator mouse models. *J Biol Chem* 279: 22276-83

38. Hinzpeter A, Lipecka J, Brouillard F, Baudoin-Legros M, Dadlez M, Edelman A, Fritsch J. 2006. Association between Hsp90 and the ClC-2 chloride channel upregulates channel function. *American Journal of Physiology-Cell Physiology* 290: C45-C56

39. Roman RM, Smith RL, Feranchak AP, Clayton GH, Doctor RB, Fitz JG. 2001. ClC-2 chloride channels contribute to HTC cell volume homeostasis. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 280: G344-G53

40. Bali M, Lipecka J, Edelman A, Fritsch J. 2001. Regulation of ClC-2 chloride channels in T84 cells by TGF-alpha. *Am J Physiol Cell Physiol* 280: C1588-98

41. Tewari KP, Malinowska DH, Sherry AM, Cuppoletti J. 2000. PKA and arachidonic acid activation of human recombinant ClC-2 chloride channels. *American Journal of Physiology-Cell Physiology* 279: C40-C50

42. Ahmed N, Ramjeesingh M, Wong S, Varga A, Garami E, Bear CE. 2000. Chloride channel activity of ClC-2 is modified by the actin cytoskeleton. *Biochem J* 352 Pt 3: 789-94

43. Bosl MR, Stein V, Hubner C, Zdebik AA, Jordt SE, Mukhopadhyay AK, Davidoff MS, Holstein AF, Jentsch TJ. 2001. Male germ cells and photoreceptors, both dependent on close cell-cell interactions, degenerate upon ClC-2Cl(-) channel disruption. *Embo Journal* 20: 1289-99

44. Clark S, Jordt SE, Jentsch TJ, Mathie A. 1998. Characterization of the hyperpolarization-activated chloride current in dissociated rat sympathetic neurons. *Journal of Physiology-London* 506: 665-78

45. Rinke I, Artmann J, Stein V. 2010. ClC-2 Voltage-Gated Channels Constitute Part of the Background Conductance and Assist Chloride Extrusion. *Journal of Neuroscience* 30: 4776-86

46. Staley K. 1994. The Role of an Inwardly Rectifying Chloride Conductance in Postsynaptic Inhibition. *Journal of Neurophysiology* 72: 273-84

47. Enz R, Ross BJ, Cutting GR. 1999. Expression of the voltage-gated chloride channel CIC-2 in rod bipolar cells of the rat retina. *Journal of Neuroscience* 19: 9841-7

48. Lan WZ, Abbas H, Lam HD, Lemay AM, Hill CE. 2005. Contribution of a time-dependent and hyperpolarization-activated chloride conductance to currents of resting and hypotonically shocked rat hepatocytes. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 288: G221-G9

49. Bouyer G, Egee S, Thomas SLY. 2007. Toward a unifying model of malaria-induced channel activity. *Proceedings of the National Academy of Sciences of the United States of America* 104: 11044-9

50. Comes N, Borras T, Morales M, Gual A, Gasull X. 2005. Identification and electrophysiological characterization of CLC-2 chloride channels in trabecular meshwork cells: Modulation by pH and cell swelling. *Investigative Ophthalmology & Visual Science* 46

51. Inagaki A, Yamaguchi S, Takahashi-Iwanaga H, Iwanaga T, Ishikawa T. 2010. Functional Characterization of a ClC-2-Like Cl- Conductance in Surface Epithelial Cells of Rat Rectal Colon. *Journal of Membrane Biology* 235: 27-41

52. Carew MA, Thorn P. 1996. Identification of CIC-2-like chloride currents in pig pancreatic acinar cells. *Pflugers Archiv-European Journal of Physiology* 433: 84-90

53. Nehrke K, Arreola J, Nguyen HV, Pilato J, Richardson L, Okunade G, Baggs R, Shull GE, Melvin JE. 2002. Loss of hyperpolarization-activated Cl- current in salivary acinar cells from Clcn2 knockout mice. *Journal of Biological Chemistry* 277: 23604-11

54. Romanenko VG, Nakamoto T, Catalan MA, Gonzalez-Begne M, Schwartz GJ, Jaramillo Y, Sepulveda FV, Figueroa CD, Melvin JE. 2008. Clcn2 encodes the hyperpolarization-activated chloride channel in the ducts of mouse salivary glands. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 295: G1058-G67

55. Blanz J, Schweizer M, Auberson M, Maier H, Muenscher A, Hubner CA, Jentsch TJ. 2007. Leukoencephalopathy upon disruption of the chloride channel ClC-2. *Journal of Neuroscience* 27: 6581-9

56. Gyomorey K, Yeger H, Ackerley C, Garami E, Bear CE. 2000. Expression of the chloride channel ClC-2 in the murine small intestine epithelium. *Am J Physiol Cell Physiol* 279: C1787-94

57. Fritsch J, Edelman A. 1996. Modulation of the hyperpolarization-activated Cl- current in human intestinal T84 epithelial cells by phosphorylation. *J Physiol* 490 (Pt 1): 115-28

58. Catalan M, Niemeyer MI, Cid LP, Sepulveda FV. 2004. Basolateral ClC-2 chloride channels in surface colon epithelium: regulation by a direct effect of intracellular chloride. *Gastroenterology* 126: 1104-14

59. Catalan M, Cornejo I, Figueroa CD, Niemeyer MI, Sepulveda FV, Cid LP. 2002. ClC-2 in guinea pig colon: mRNA, immunolabeling, and functional evidence for surface epithelium localization. *Am J Physiol Gastrointest Liver Physiol* 283: G1004-13

60. Inagaki A, Yamaguchi S, Takahashi-Iwanaga H, Iwanaga T, Ishikawa T. 2010. Functional characterization of a ClC-2-like Cl(-) conductance in surface epithelial cells of rat rectal colon. *J Membr Biol* 235: 27-41

61. Bosl MR, Stein V, Hubner C, Zdebik AA, Jordt SE, Mukhopadhyay AK, Davidoff MS, Holstein AF, Jentsch TJ. 2001. Male germ cells and photoreceptors, both

dependent on close cell-cell interactions, degenerate upon ClC-2 Cl(-) channel disruption. *EMBO J* 20: 1289-99

62. Lipecka J, Bali M, Thomas A, Fanen P, Edelman A, Fritsch J. 2002. Distribution of ClC-2 chloride channel in rat and human epithelial tissues. *Am J Physiol Cell Physiol* 282: C805-16

63. Cuppoletti J, Malinowska DH, Tewari KP, Li QJ, Sherry AM, Patchen ML, Ueno R. 2004. SPI-0211 activates T84 cell chloride transport and recombinant human ClC-2 chloride currents. *Am J Physiol Cell Physiol* 287: C1173-83

64. Catalan MA, Flores CA, Gonzalez-Begne M, Zhang Y, Sepulveda FV, Melvin JE. 2012. Severe defects in absorptive ion transport in distal colons of mice that lack ClC-2 channels. *Gastroenterology* 142: 346-54

65. Pena-Munzenmayer G, Catalan M, Cornejo I, Figueroa CD, Melvin JE, Niemeyer MI, Cid LP, Sepulveda FV. 2005. Basolateral localization of native ClC-2 chloride channels in absorptive intestinal epithelial cells and basolateral sorting encoded by a CBS-2 domain di-leucine motif. *J Cell Sci* 118: 4243-52

66. Mohammad-Panah R, Gyomorey K, Rommens J, Choudhury M, Li C, Wang Y, Bear CE. 2001. ClC-2 contributes to native chloride secretion by a human intestinal cell line, Caco-2. *J Biol Chem* 276: 8306-13

67. Jakab RL, Collaco AM, Ameen NA. 2012. Lubiprostone targets prostanoid signaling and promotes ion transporter trafficking, mucus exocytosis, and contractility. *Dig Dis Sci* 57: 2826-45

68. Lacy BE, Levy LC. 2007. Lubiprostone: a chloride channel activator. *J Clin Gastroenterol* 41: 345-51

69. Ueno R, Osama H, Habe T, Engelke K, Patchen M. 2004. Oral SPI-0211 increases intestinal fluid secretion and chloride concentration without altering serum electrolyte levels. *Gastroenterology* 126: A298-A

70. Fei G, Raehal K, Liu S, Qu MH, Sun X, Wang GD, Wang XY, Xia Y, Schmid CL, Bohn LM, Wood JD. 2010. Lubiprostone reverses the inhibitory action of morphine on intestinal secretion in guinea pig and mouse. *J Pharmacol Exp Ther* 334: 333-40

71. Barish CF, Drossman D, Johanson JF, Ueno R. 2010. Efficacy and Safety of Lubiprostone in Patients with Chronic Constipation. *Digestive Diseases and Sciences* 55: 1090-7

72. Drossman DA, Chey WD, Johanson JF, Fass R, Scott C, Panas R, Ueno R. 2009. Clinical trial: lubiprostone in patients with constipation-associated irritable bowel syndrome - results of two randomized, placebo-controlled studies. *Alimentary Pharmacology & Therapeutics* 29: 329-41

73. Schey R, Rao SSC. 2011. Lubiprostone for the Treatment of Adults with Constipation and Irritable Bowel Syndrome. *Digestive Diseases and Sciences* 56: 1619-25

74. Bao HF, Liu L, Self J, Duke BJ, Ueno R, Eaton DC. 2008. A synthetic prostone activates apical chloride channels in A6 epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 295: G234-51

75. Bijvelds MJ, Bot AG, Escher JC, De Jonge HR. 2009. Activation of intestinal Cl- secretion by lubiprostone requires the cystic fibrosis transmembrane conductance regulator. *Gastroenterology* 137: 976-85

76. Ao M, Venkatasubramanian J, Boonkaewwan C, Ganesan N, Syed A, Benya RV, Rao MC. 2011. Lubiprostone activates Cl- secretion via cAMP signaling and increases membrane CFTR in the human colon carcinoma cell line, T84. *Dig Dis Sci* 56: 339-51

77. Norimatsu Y, Moran AR, MacDonald KD. 2012. Lubiprostone activates CFTR, but not ClC-2, via the prostaglandin receptor (EP(4)). *Biochem Biophys Res Commun* 426: 374-9

78. Schiffhauer ES, Vij N, Kovbasnjuk O, Kang PW, Walker D, Lee S, Zeitlin PL. 2013. Dual activation of CFTR and CLCN2 by lubiprostone in murine nasal epithelia. *Am J Physiol Lung Cell Mol Physiol* 304: L324-31

79. Sweetser S, Busciglio IA, Camilleri M, Bharucha AE, Szarka LA, Papathanasopoulos A, Burton DD, Eckert DJ, Zinsmeister AR. 2009. Effect of a chloride channel activator, lubiprostone, on colonic sensory and motor functions in healthy subjects. *Am J Physiol Gastrointest Liver Physiol* 296: G295-301

80. Sun X, Wang X, Wang GD, Xia Y, Liu S, Qu M, Needleman BJ, Mikami DJ, Melvin WS, Bohn LM, Ueno R, Wood JD. 2011. Lubiprostone reverses the inhibitory action of morphine on mucosal secretion in human small intestine. *Dig Dis Sci* 56: 330-8

81. De Lisle RC, Mueller R, Roach E. 2010. Lubiprostone ameliorates the cystic fibrosis mouse intestinal phenotype. *BMC Gastroenterol* 10: 107

82. De Lisle RC. 2012. Lubiprostone stimulates small intestinal mucin release. *BMC Gastroenterol* 12: 156

83. Bassil AK, Borman RA, Jarvie EM, McArthur-Wilson RJ, Thangiah R, Sung EZ, Lee K, Sanger GJ. 2008. Activation of prostaglandin EP receptors by lubiprostone in rat and human stomach and colon. *Br J Pharmacol* 154: 126-35

84. Cuppoletti J, Malinowska DH, Chakrabarti J, Ueno R. 2008. Effects of lubiprostone on human uterine smooth muscle cells. *Prostaglandins Other Lipid Mediat* 86: 56-60

85. Chan WW, Mashimo H. 2013. Lubiprostone Increases Small Intestinal Smooth Muscle Contractions Through a Prostaglandin E Receptor 1 (EP1)-mediated Pathway. *J Neurogastroenterol Motil* 19: 312-8

86. MacVinish LJ, Cope G, Ropenga A, Cuthbert AW. 2007. Chloride transporting capability of Calu-3 epithelia following persistent knockdown of the cystic fibrosis transmembrane conductance regulator, CFTR. *Br J Pharmacol* 150: 1055-65

87. MacDonald KD, McKenzie KR, Henderson MJ, Hawkins CE, Vij N, Zeitlin PL. 2008. Lubiprostone activates non-CFTR-dependent respiratory epithelial chloride secretion in cystic fibrosis mice. *Am J Physiol Lung Cell Mol Physiol* 295: L933-40

88. Fei G, Wang YZ, Liu S, Hu HZ, Wang GD, Qu MH, Wang XY, Xia Y, Sun X, Bohn LM, Cooke HJ, Wood JD. 2009. Stimulation of mucosal secretion by lubiprostone (SPI-0211) in guinea pig small intestine and colon. *Am J Physiol Gastrointest Liver Physiol* 296: G823-32

89. Joo NS, Wine JJ, Cuthbert AW. 2009. Lubiprostone stimulates secretion from tracheal submucosal glands of sheep, pigs, and humans. *Am J Physiol Lung Cell Mol Physiol* 296: L811-24

90. Mizumori M, Akiba Y, Kaunitz JD. 2009. Lubiprostone stimulates duodenal bicarbonate secretion in rats. *Dig Dis Sci* 54: 2063-9

91. Cuthbert AW. 2011. Lubiprostone targets prostanoid EP(4) receptors in ovine airways. *Br J Pharmacol* 162: 508-20

92. Musch MW, Wang Y, Claud EC, Chang EB. 2013. Lubiprostone decreases mouse colonic inner mucus layer thickness and alters intestinal microbiota. *Dig Dis Sci* 58: 668-77

CHAPTER 3

PHARMACEUTICAL ACTIVATION OR GENETIC ABSENCE OF CLC-2 ALTERS TIGHT JUNCTIONS DURING EXPERIMENTAL COLITIS

ABSTRACT

We have previously reported that the ClC-2 chloride channel has an important role in the regulation of tight junction barrier function during experimental colitis, and the pharmaceutical ClC-2 activator lubiprostone initiates intestinal barrier repair in ischemic-injured intestine. Thus, we hypothesized that pharmaceutical ClC-2 activation would have a protective and therapeutic effect in murine models of colitis which would be absent in ClC-$2^{-/-}$ mice. We administered lubiprostone to wild type or ClC-$2^{-/-}$ mice with dextran sulfate sodium (DSS) or 2, 4, 5-trinitrobenxene sulfonic acid (TNBS)-induced colitis. We determined the severity of colitis and assessed intestinal permeability. Selected tight junction proteins were analyzed by western blotting and immunofluorescence/confocal microscopy, whereas proliferative and differentiated cells were examined with special staining and immunohistochemistry. Oral preventive or therapeutic administration of lubiprostone significantly reduced the severity of colitis and reduced intestinal permeability in both DSS and TNBS-induced colitis. Preventive treatment with lubiprostone induced significant recovery of the expression and distribution of selected sealing tight junction proteins in mice with DSS-induced colitis. In addition, lubiprostone reduced crypt proliferation and increased the number of differentiated epithelial cells. Alternatively, when lubiprostone was administered to ClC-$2^{-/-}$ mice, the protective effect against DSS colitis was limited. This study suggests a central role for ClC-2 in restoration of barrier function and tight junction architecture in experimental murine colitis, which can be therapeutically targeted with lubiprostone.

INTRODUCTION

Intestinal homeostasis requires an epithelial barrier that separates the luminal contents from the interstitium. [1] Increased intestinal paracellular permeability, or barrier dysfunction, is thought to be a major factor in the pathogenesis of inflammatory bowel diseases (IBD), which include ulcerative colitis (UC) and Crohn's disease (CD). [2] Disruption of intestinal barrier function has been detected in experimental IBD models. [3 4] The intestinal epithelial barrier consists of a highly organized complex of intercellular junctions, within which the tight junction is the most critical for regulating barrier function. [5] The tight junction is the most apical component of the epithelial junctional complex, acting as a barrier (gate) to the paracellular pathway by regulating passive diffusion of solutes and macromolecules and as a fence that restricts the movement of lipids and membrane proteins between the apical and basolateral membrane of polarized intestinal epithelial cells. [6] Moreover, the intestinal tight junctions are thought to play a role in regulating epithelial proliferation and differentiation, as well as overall mucosal architecture.[7] In addition, goblet cells produce mucin, covering the entire length of the gastrointestinal tract, which further protects the mucosal surface from harmful molecules and bacteria, and reinforces the overall intestinal barrier. [8] Colonic mucosa from IBD patients and from animals with experimental colitis have shown structural alteration of the epithelial barrier, including a reduced number of horizontal tight junction strands on freeze-fracture electromicroscopy, altered tight junction protein expression and subcellular distribution, and a decreased number of goblet cells resulting in reduction of mucin secretion. [9-13]

The ClC-2 chloride channel is expressed in the plasma membranes of epithelial cells from many mammalian tissues. [14] The physiological contribution of ClC-2 in the intestine is not completely understood. However, in our previous work, we have demonstrated a critical role for ClC-2 in epithelial repair. [15-20] Localization of ClC-2 to the region of the tight junction in porcine and murine intestine is associated with its regulatory interactions with signaling molecules in the tight junction complex, resulting in hastened re-sealing of the paracellular space. [18 20-22] In previous studies, we have shown that ClC-2 knockout mice have retarded recovery of barrier function following small intestinal ischemic injury, and knockdown of ClC-2 in Caco-2 cells reduces the ability of cultured cells to form tight junction complexes. [16 18] More recently, we reported that ClC-2 has a critical role in regulation of tight junctions in experimental colitis and its expression is significantly reduced in the colon of patients with UC. [29]

Lubiprostone is a synthetic prostone ClC-2 activator, inducing secretion and is approved for the treatment of chronic idiopathic constipation (CIC), irritable bowel syndrome with constipation (IBS-C), and opioid induced constipation (OIC).[23 24] Previously, we reported that lubiprostone initiates intestinal barrier repair in ischemic-injured intestine via its principal target, ClC-2. [19] In the present study, we investigated the possible therapeutic effects of oral treatment with lubiprostone in murine experimental colitis, and determined whether this effect was dependent on ClC-2. Our findings indicate that oral administration of lubiprostone dramatically improves mucosal and tight junction architecture, with associated benefits on barrier function, in a ClC-2-dependent manner. In addition, we also noted a novel

action for lubiprostone; that of an increased proportion of goblet cells, which occurred in a ClC-2-independent manner.

METHODS

Animals

Studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Female C57BL/6 (6-week-old) and BALB/c (7-week-old) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and breeding pairs of heterozygous mice (ClC-$2^{+/-}$) were a kind gift of Dr. James E. Melvin (University of Rochester, NY, USA). The generation of ClC-2 null (ClC-$2^{-/-}$) mice and genotyping PCR has been previously described. [18 25]

Induction and Assessment of Murine colitis

DSS colitis was induced in C57BL/6 mice by offering 3% (w/v) DSS (36,000 - 50,000 daltons; MP Biomedicals, CA, USA) in autoclaved drinking water for 5 days. At the end of day 5, mice were returned to normal drinking water until day 8 when they were euthanized. TNBS colitis was induced by the intrarectal administration of 2.5% TNBS (Sigma-Aldrich, MO, USA) in 50% ethanol to BALB/c mice. The mice were lightly anesthetized with isoflurane (Aerrne, Baxter Healthcare Corporation, IL, USA); after which a 3.5 Fr umbilical catheter (Utah Medical Product, Inc., Utah, USA) lubricated with lubricating jelly was inserted into the colon through the anus. An enema of 100 μL of 2.5% (v/v) TNBS was injected when the tip of the catheter was 4 cm inside the anus. The mice were held in a vertical position for 1 minute after the intrarectal injection. The control group received 100 μL of 50% ethanol in a similar manner. Body weight and clinical signs of colitis were

monitored daily, and the disease activity index (DAI) was determined, as previously described. [26] At the end of the experiments, the colons were excised and colon length measurements were performed. In addition, the distal portion of each colon was immediately fixed in 10% neutral buffered formalin solution, embedded in paraffin wax and then sectioned before being deparaffinized. Slides were stained using H&E standard techniques and the microscopic score was determined as described previously. [27]

Pharmacological Treatments.

The following series of treatments were designed to investigate the preventive and/ or the therapeutic properties of lubiprostone (Sucampo Pharmaceuticals, Inc., MD, USA) in DSS- and TNBS-induced colitis in mice. DSS colitis mice were orally treated by gavage with 1 (low), 10 (middle) or 100 (high) μg/kg lubiprostone from day 0 to day 8 (preventive treatment) or with 10 or 100 μg/kg from day 3 to day 7 (therapeutic treatment). To determine the therapeutic dose of prostones, animal equivalent dose (10 μg/kg) is calculated using the following formula. Animal equivalent dose = Human dose x Human K_m ÷ Animal K_m.[28] TNBS colitis mice were also orally administered lubiprostone by gavage with 100 μg/kg from day 0 to day 3. To explore the dependency of lubiprostone on ClC-2, another set of experiments was performed in ClC-$2^{-/-}$ mice gavaged with 100 μg/kg of lubiprostone from day 0 to day 8. Negative control and colitis control groups received only medium chain triacylglyceride (MCT, Sucampo Pharmaceuticals, Inc., MD, USA) vehicle solution.

***In vivo* Intestinal Permeability Assay.**

In vivo intestinal permeability was assessed by luminal enteral administration of FITC-dextran (4,000 daltons; Sigma-Aldrich, MO, USA), a non-metabolizable

macromolecule that is used as a permeability probe. Food was withdrawn for 3 h and mice were gavaged with FITC-dextran (6 mg/10 g body weight) 2 hours before euthanasia. Whole blood was collected by cardiac puncture at the time of euthanasia. Fluorescence intensity in serum was analyzed using a plate reader (excitation, 492 nm; emission, 525 nm). The concentration of FITC-dextran was determined from FITC-dextran standard curve generated by serial dilution. Permeability was calculated by linear regression of sample fluorescence.

Real time PCR for cytokine expression.

Total RNA was isolated from distal colonic tissues from mice with DSS colitis in the preventive lubiprostone treatment group using an RNeasy kit (Qiagen, CA, USA) and quantified spectrophotometrically. Equal amounts of RNA were used for complementary DNA synthesis (SuperScript III First-Strand Synthesis; Invitrogen, CA, USA). Real time PCR (RT-PCR) was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc., CA, USA), the ABI StepOnePlus system (Applied Biosciences, CA, USA), and PCR primers obtained from the Integrated DNA Technology, Inc. (IA, USA). (Table 1)

Gel Electrophoresis and Western Blotting

Distal colonic tissues from DSS colitis WT and ClC-2$^{-/-}$ mice were lysed in Tissue Extraction Reagent I (Invitrogen, CA, USA), homogenized and centrifuged. Supernatants were quantified applying a BCA protein assay kit (Thermo Scientific, IL, USA). Proteins were separated 4-12% Bis-Tris gels with XT MOPS SDS running buffer (both Bio-Rad Laboratories, Inc., CA, USA). After protein transfer to a polyvinylidene difluoride membrane, the membranes were probed using anti-ZO-1, anti-occludin, anti-claudin-1, and

anti-claudin-2 (Invitrogen, CA, USA) antibodies. Horseradish peroxidase (HRP)-conjugated secondary antibodies were used (Santa Cruz Biotechnology, Inc., TX, USA).

Immunofluorescence Analysis.

Distal colonic cryosections from WT and ClC-2$^{-/-}$ mice were fixed for 10 minutes with cold acetone at -20 °C. Immunostaining was performed using primary antibodies: anti-ZO-1, anti-occludin, anti-claudin-1, anti-claudin-2 (Invitrogen, CA, USA), and FITC-EpCAM (BioLegend, CA, USA). Cy3 conjugated anti-immunoglobulin G was used as a secondary antibody (Invitrogen, CA, USA). After nuclear staining by To-Pro-3 (Invitrogen, CA, USA), slides were mounted in fluorescent mounting media (DakoCytomation, CA, USA) an examined with a Nikon Eclipse 2000E inverted microscope equipped with Nikon C1 confocal laser scanning system using EZ-C1 software. We only used intact structure area with normal distribution of EpCAM, a membranous marker, to compare the immunolocalization of tight junction proteins.

Proliferative/differentiated cell analysis

Colonic crypt height quantification was performed using the Image J software. 20 random colonic crypts were measured from each H&E stained section, to make n=1. All measurements were performed in a blinded fashion.

Immunohistochemistry for proliferating cell nuclear antigen (PCNA), carbonic anhydrase II (CA II; colonocyte), and chromogranin A (CgA; enterochromaffin cell) on murine colonic tissues were performed by standard methods. Heat-activated antigen retrieval was performed in sodium citrate buffer (pH6). Paraffin sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in

methanol for 10 minutes. After blocking in Protein Block Serum-Free (Dako, CA, USA), the sections were incubated in primary antibodies; mouse anti-PCNA (Abcam, MA, USA), goat anti-CAII (Santa Cruz Biotechnology, inc., TX, USA), and rabbit anti-CgA (ImmunoStar, Inc. WI, USA). After washing, the EnVision™ system (Dako, CA, USA) was employed to reveal the reaction. After washing slides were developed for 5 min with 3, 3'-diaminobenzindin chromogen and counterstained with hematoxylin. 10 intact crypts were randomly analyzed for PCNS or CgA positive cells among three to four colonic cross-sections from each mouse to make n = 1. The results were calculated as the percentage of total crypt cells.

WT and ClC-2$^{-/-}$ mice sections were incubated with alcian blue solution (1% w/v alcian blue, 8GX in 3% v/v acetic acid, pH2.5) and washed with diH_2O. Sections were counterstained with nuclear fast red (0.1% w/v nuclear fast red, 5% w/v aluminum sulfate in diH_2O). Slides were dehydrated and mounted. Goblet cell numbers were quantified from 15 random colonic crypts glands from each mouse.

Statistical Analysis

Data are reported as mean ± standard error. Differences between groups were tested with one-way ANOVA and LSD post-hot testing (IBM SPSS Statistics 21). Where appropriate, differences between two groups were tested with a Student's t-test (Excel, Microsoft 2013).

RESULTS

The ClC-2 activator lubiprostone reduces DSS-induced colitis in a preventive and therapeutic manner

The administration of DSS solution resulted in reproducible colitis in mice, which resembles the acute phase of human UC, and is characterized by increased DAI and colonic damage. [26] To assess the potential effect of the ClC-2 activator lubiprostone on DSS-induced colitis, mice were treated orally by gavage with three different doses (1, 10, and 100μg/kg) once a day from day 0 to day 8 (preventive treatment) to detect potential dose-dependent effects. DSS-treated C57BL/6 mice started to lose weight on day 6 and lost ~11% of their body weight by day 8. No weight loss was seen in the control groups, which had access to tap water without DSS. Preventive oral treatment with 100μg/kg of lubiprostone but not 1 or 10μg/kg significantly protected from marked body weight loss (Fig. 1A). Alternatively, all dosages of lubiprostone significantly reduced the DAI in a dose-dependent manner (Fig. 1B). In order to determine if lubiprostone had an effect on intestinal paracellular permeability, as we have shown in previous studies, *in vivo* FITC-dextran permeability assays were performed. These experiments revealed that DSS colitis mice receiving oral treatment with 100μg/kg of lubiprostone had significantly decreased intestinal permeability to FITC conjugated-dextran compared with untreated DSS colitis mice (Fig. 1C). Administration of 10 or 100μg/kg of lubiprostone dose-dependently prevented reductions in colon length ($P<0.05$, Fig. 1D and 1E). Histological evaluation of colonic mucosa from mice subjected to DSS in their water revealed transmural infiltration with inflammatory cells, associated with epithelial erosions and severe edema in the submucosa. Histological injury scores were also dose-dependently reduced by administration of lubiprostone at doses of 10 or 100μg/kg, with reduced granulocyte infiltration and submucosal edema (Fig. 1F).

In further experiments designed to test the potential therapeutic effects of lubiprostone, we noted that mice with DSS-induced acute colitis had body weight loss and marked diarrhea with bloody stools, resulting in a sharp increase of the DAI from day 3 onwards. Therefore, therapeutic treatment was instituted from day 3 to day 8 with lubiprostone doses of 10μg/kg and 100μg/kg. These studies revealed that the high dose lubiprostone significantly reduced clinical and pathological evidence of DSS colitis, such as body weight loss, DAI, colon shortening, histologic score, and intestinal permeability (Fig. 2). These effects were not apparent at the lower lubiprostone dose of 1μg/kg. Taken together, these data indicate that oral treatment of mice with lubiprostone has preventive and therapeutic effects on experimental colitis, although preventive effects were noted at lower dosages as compared to therapeutic dosages.

Preventive administration of lubiprostone decreases colonic inflammatory mediators in mice with DSS colitis

To examine whether the protection from DSS-induced colitis in mice pre-treated with lubiprostone was associated with a decrease in the production of inflammatory mediators, the mRNA levels of the cytokines TNF-α, IFN-γ, and IL-1β were assessed in colonic tissues using real-time PCR (Table 1). Mice with DSS-induced colitis had significant increases in the pro-inflammatory cytokines TNF-α, IFN-γ, and IL-1β. In mice receiving preemptive oral lubiprostone (100μg/kg), there were significant reductions in expression of TNF-α and IL-1β mRNA but not of IFN- γ mRNA (Fig. 3). In addition, we measured the colonic mRNA expression of the anti-inflammatory mediators IL-10 and TGF-β, and showed significantly increased IL-10 but not TGF-β levels in DSS colitis tissues compared to the control group.

Interestingly, lubiprostone significantly decreased both IL-10 and TGF-β levels in the distal colonic tissue of mice with DSS-induced colitis (Fig. 3), which may have been attributable to an overall reduction in inflammation in the presence of lubiprostone, possibly reducing the impetus for anti-inflammatory signaling.

Preventive treatment with lubiprostone protects tight junction structure in DSS-induced colitis

Tight junctions are assembled at the apical-lateral cellular domain, where they form the most critical component of the paracellular barrier. [29] In prior experiments, we had already shown that this paracellular barrier was disturbed in DSS colitis, based on changes in permeability assays. Therefore, to determine if the action of lubiprostone in DSS-induced colitis mice is linked to the tight junction, we initially determined expression of the tight junction proteins ZO-1, occludin, claudin-1, and claudin-2 by western blot. Expression of ZO-1, claudin-1, and claudin-2 was not different in the distal colon of colitis mice as compared to control mice. However, occludin expression was decreased in colonic mucosa from mice with DSS-induced colitis. Alternatively, gavaging mice with lubiprostone (100μg/kg) prior to administration of DSS resulted in significant increases in occludin and claudin-1 expression. Furthermore, expression of claudin-2, a pore-forming tight junction protein, was significantly reduced in colitis mice receiving lubiprostone as compared to controls (Fig. 4A and B).

Given the importance of the cellular localization of tight junctions in the regulation of barrier function, the distribution of tight junction proteins was visualized by immunofluorescence confocal microscopy. In the control group, immunolocalization of tight

junction proteins was observed primarily in the intercellular junctions of the epithelial cells adjacent to the apical membrane. The distribution of tight junction proteins was altered by DSS administration, including an apparent reduction in occludin fluorescence, claudin-1 endocytosis, and increased claudin-2 fluorescence consistent with our findings on western analyses. The distribution of ZO-1 and EpCAM (a membrane marker) was maintained in colitis mice. Interestingly, although claudin-2 fluorescence was primarily localized to the crypt in control colon, its expression was increased in both the mucosal surface and crypt epithelium in DSS colitis mice. Preemptive administration of lubiprostone resulted in recovery of tight junction immunolocalization of occludin, claudin-1, and claudin-2 in DSS-induced colitis (Fig. 4C). Collectively, these data indicate that treatment with lubiprostone results in maintenance of tight junction protein expression and distribution in DSS-induced colitis.

Lubiprostone has limited protective effects in DSS-induced ClC-2$^{-/-}$ mice colitis

Considering the purported action of lubiprostone as a ClC-2 activator, we next investigated the effect of lubiprostone on colitis in ClC-2 knockout mice. Cuppoletti et al. [30] showed that lubiprostone is a selective and dose-dependent activator of ClC-2, with no observable effect on CFTR. However, other studies have suggested alternate actions of lubiprostone, clouding the issue of the mechanisms of action for this prostone. [31-34] To clarify the mechanism of action of lubiprostone in our murine DSS colitis model, we performed a study using lubiprostone pretreatment with 100μg/kg lubiprostone in ClC-2$^{-/-}$ mice. Oral pretreatment with lubiprostone did not protect against colon shortening or histological evidence of colitis, as had been the case in lubiprostone-treated wild type mice

with DSS-induced colitis. However, pretreatment with lubiprostone did provide some protection in ClC-2$^{-/-}$ mice from clinical signs of colitis, including less marked reductions in body weight, amelioration of increases in DAI, and reductions in intestinal permeability (Fig. 5). However, the level of protection was notably less than that noted in lubiprostone-treated wild type mice. In addition, preemptive treatment with lubiprostone did not change expression and distribution of tight junction proteins (ZO-1, occludin, claudin-1, and -2) in DSS-induced ClC-2$^{-/-}$ mice colitis model (Fig. 6). To determine the effect of an intermediary dose of lubiprostone (10μg/kg) given preemptively, we performed an additional trial on ClC-2$^{-/-}$ mice with DSS-induced colitis. However, 10μg/kg lubiprostone did not protect against clinical signs, colon shortening, and histological score in ClC-2$^{-/-}$ mice with colitis (Fig. 7), whereas 10μg/kg lubiprostone had protective effects on wild type mice with DSS-induced colitis (Fig. 1). Therefore, these results indicated that the major protective effect of lubiprostone in DSS-induced colitis is ClC-2 dependent.

Alternate protective effects of lubiprostone pretreatment

Although ClC-2$^{-/-}$ colitis mice treated with 100μg/kg lubiprostone did not differ from untreated colitis mice in their immune cell infiltration, epithelial damage, and tight junction structure, histological evaluation suggested changes in the goblet cell number. Interestingly, mice with DSS colitis have been reported to have evidence of increased crypt cell proliferation with concurrent reductions in the number of differentiated epithelial cells [35]. Therefore, to find alternative protective mechanisms for lubiprostone, we performed immunohistochemistry for proliferating and differentiated cells, as well as Alcian blue staining for mucins in colonic tissues of ClC-2$^{-/-}$ colitis mice. There were no differences in

crypt height, the number of proliferative cells ($PCNA^+$), colonocytes ($CA\ II^+$), and enterochromaffin cells (CgA^+) between the two groups (Fig. 8A-G). However, the number of goblet cells was significantly increased in lubiprostone-treated colitis mice compared with $ClC\text{-}2^{-/-}$ colitis mice (Fig. 8Hand I). These findings suggested that the modest protective effects of 100μg/kg lubiprostone in $ClC\text{-}2^{-/-}$ mice might be attributable to a change in the mucus-secreting capacity of the mucosa. To further explore these findings, additional analyses of tissues from wild type colitis with DSS colitis were performed. In these mice, DSS colitis was associated with an increase in crypt height, proliferation index (increased $PCNA^+$ cells), and a reduction in $CAII^+$, CgA^+ cells, and goblet cells (Fig. 9A-I). Treatment of wild type DSS colitis mice with 100μg/kg lubiprostone significantly reversed all of these trends. Thus, high dose lubiprostone has significant effects on goblet cell numbers, as had been seen in $ClC\text{-}2^{-/-}$ mice. However, in wild type mice there was also an effect of lubiprostone on epithelial proliferation/differentiation, which had not been seen in the absence of ClC-2. Thus, for the full effect of lubiprostone on proliferation and cell differentiation, ClC-2 appeared to be required.

Lubiprostone ameliorates murine colitis induced by TNBS.

In order to extend our observations on the beneficial effects of lubiprostone in DSS colitis, a TNBS-induced model of CD in BALB/c mice was used. TNBS-induced colitis mice had progressive reductions in body weight, and increases in DAI that were maximal at day 3, associated with marked histological pathology, including mucosal infiltration with neutrophils and lymphocytes, ulceration and severe submucosal edema. However, mice treated orally with lubiprostone (100 μg/kg) showed significantly less loss of body weight

loss and a significantly reduced DAI (Fig. 10A and B). As had been shown in mice with DSS colitis, lubiprostone also significantly ameliorated colon shortening induced by TNBS (Fig. 10C) and restored the histological score compared with vehicle control mice (Fig. 10E). In terms of barrier function, lubiprostone also significantly reduced TNBS-induced increases in intestinal permeability (Fig. 10D).

DISCUSSION

In the present report, pharmaceutical targeting of the chloride channel ClC-2 appears to reduce symptoms in experimental colitis, associated with restoration of barrier function. The expression of ClC-2 in the apical lateral membrane raises questions as to its regulatory interactions with signaling molecules in the tight junctions. [21 36 37] Previously, we have demonstrated that the severity of colitis and intestinal permeability is increased in ClC-$2^{-/-}$ mice, associated with perturbed mucosal epithelial tight junctions. [15] In a detailed mechanistic study, we have demonstrated that ClC-2 plays a critical role in shuttling the tight junction protein occludin to the apical lateral membrane via an interaction with caveolin-1 and the small GTPase Rab5. [16] We have also shown that the ClC-2 activator lubiprostone accelerates barrier repair in ischemic-injured porcine intestine by regulating tight junctions in a ClC-2-dependent manner. [19 38] In the present study, we used two distinct murine models of IBD: DSS- and TNBS-induced colitis. Oral administration of lubiprostone in a preventive manner prior to administration of DSS reduced mucosal pathology and abnormal barrier function in DSS-induced colitis, a widely used model for UC-like intestinal inflammation, and preserved intestinal barrier integrity via ClC-2-dependent tight junction reconstitution. Moreover, lubiprostone treatment suppressed the corresponding intestinal inflammation,

leukocyte infiltration and overproduction of pro-inflammatory cytokines observed during colitis. Lubiprostone also showed therapeutic effects when mice were treated after showing clinical signs of DSS-induced colitis. In addition, oral administration of a relatively high dose of lubiprostone (100 μg/kg) protected mice against TNBS-induced colitis, which more closely resembles human CD. Although DSS- and TNBS-induced colitis models have distinct differences from human IBD, these models share many clinical and pathological features with human IBD with regard to loss of barrier function and inflammatory response. [27 39]

Intestinal permeability has been proposed to be primarily under the control of interepithelial tight junctions. [1] In the present study, we show that preventive treatment with lubiprostone prevented DSS-induced alteration of tight junction proteins in wild type mice but not ClC-2$^{-/-}$ mice. In a previous study examining human IBD tissues, a significant reduction in the expression of tight junction molecule occludin was detected at the protein and mRNA levels by western and northern blotting respectively in colonic epithelial cells. [40] Our present results also showed that the total and apical membrane expression of occludin were diminished in DSS-induced colitis. However, a high dose of lubiprostone induced restoration of occludin expression and localization in murine DSS colitis. Several studies have shown that expression of the pore forming tight junction protein claudin-2 was significantly elevated in active IBD. [41 42] This over-expression of claudin-2 is thought to contribute to the defect in barrier function in IBD. [43] In the present study, although the total expression of claudin-2 was not significantly increased in tissues from mice with DSS-induced colitis, the apical membrane expression of claudin-2 was dramatically increased in

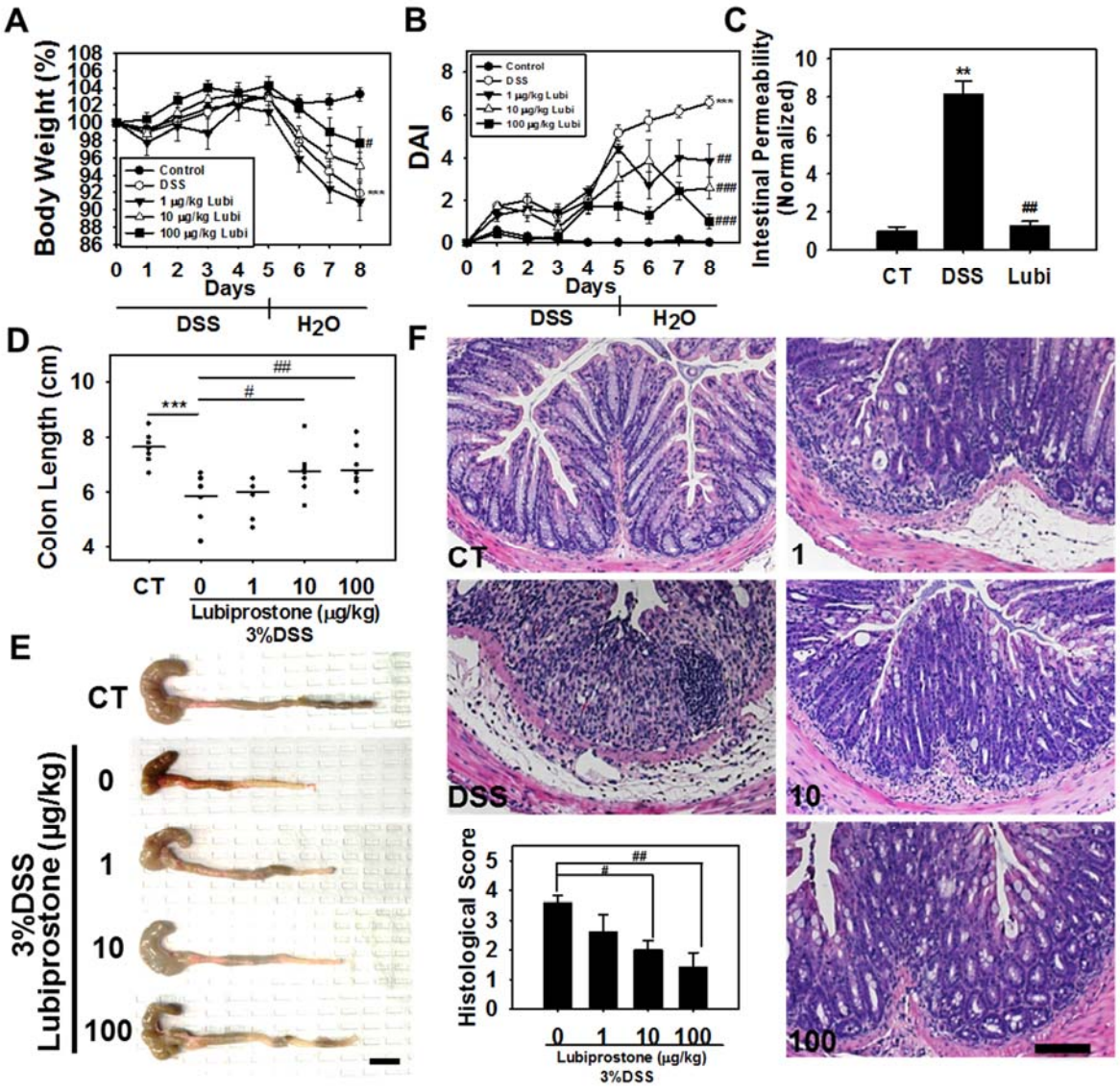
the mucosal surface and crypts of colitis mice. Oral treatment with lubiprostone significantly reduced the total and apical membrane expression of claudin-2, suggesting a direct treatment effect relevant to human IBD. Regarding other tight junction proteins in IBD, one report indicated that total expression of the sealing junctional protein claudin-1 was significantly decreased in patients with UC. [44] Although total expression of claudin-1 was not significantly changed in DSS-induced colitis in the present study, claudin-1 endocytosis was detected by immunofluorescence. However, lubiprostone treatment restored immunolocalization of claudin-1 in mice with DSS-induced colitis as further evidence of a positive mechanistic effect of this ClC-2 activator. ClC-$2^{-/-}$ mice with DSS colitis also showed redistribution of tight junction proteins similar to wild type mice with colitis. However, oral treatment with high dose lubiprostone did not have any effect on tight junction proteins in these knockout animals. These data are therefore highly suggestive of the need for ClC-2 as a target for lubiprostone in order to repair the tight junction barrier in the setting of murine DSS colitis.

Although most studies concerning the role of tight junctions in IBD have focused on their critical role in regulating paracellular permeability, tight junctions are also mechanistically involved in regulation of cell proliferation and differentiation. [45] Some of this knowledge has come from genetically modified mice. For example, claudin-15 deficiency leads to megaintestine caused by an increased number of transit-amplifying intestinal cells in the crypts. [46] Additionally, it has been reported a number of claudins are significantly altered in colitis-associated colorectal cancer, supporting the existence of a relationship between the tight junctions and intestinal epithelial cell proliferation. [47] In our

present study, ClC-2-dependent tight junction reconstitution of lubiprostone reduced epithelial proliferation and induced differentiation in experimental colitis. The observed effects of lubiprostone-induced goblet cell proliferation have been not previously been noted to our knowledge, and while we speculate proliferation may relate to changes in tight junction structure, we have not investigated these mechanisms in detail.

Overall, our data demonstrate that the ClC-2 activator lubiprostone ameliorates both DSS- and TNBS- induced colitis in mice. The beneficial effects of lubiprostone were associated with the protection of tight junctions as well as regulation of cellular differentiation that were dependent on the presence of ClC-2 (Fig. 11). Alternatively, lubiprostone promoted goblet cell proliferation in DSS-induced colitis in ClC-$2^{-/-}$ mice resulting in limited protective effects on intestinal barrier function. Although lubiprostone is presently indicated as a pro-secretory drug in a number of human conditions involving constipation, [23 24] pharmacologically targeting ClC-2 also appears to restore tight junction barrier function in experimental colitis, and therefore may provide a mechanistic basis for future exploration of alternative therapies of IBD.

Figure 1. **Protective effect of lubiprostone in DSS-induced colitis mice.** A. and B. Effect of lubiprostone on body weight (A) and disease activity index (B) in DSS-induced colitis. C. Effect of 100 μg/kg of lubiprostone on intestinal permeability in DSS-induced colitis. (n=6) D. and E. Effect of orally treated lubiprostone on colon shortening in DSS-induced colitis (n=9). Bar=1 cm. F. Histologic scoring and representative H&E-stained sections from colonic tissue of each groups (n=5). Bar=100 μm Data are represented as means ± SEM. **p* <0.05, ***p* <0.01, and *** *p* <0.001 vs. controls (CT); # *p* <0.05, ## *p* <0.01, and ### *p* <0.001 vs. DSS without lubiprostone; one-way ANOVA


A
Body Weight (%)
Control
DSS
1 μg/kg Lubi
10 μg/kg Lubi
100 μg/kg Lubi
Days
DSS
H2O
B
DAI
Days
DSS
H2O
C
Intestinal Permeability (Normalized)
CT
DSS
Lubi
D
Colon Length (cm)
CT
0
1
10
100
Lubiprostone (μg/kg)
3%DSS
E
CT
3%DSS Lubiprostone (μg/kg)
0
1
10
100
F
CT
1
DSS
10
100
Histological Score
Lubiprostone (μg/kg)
3%DSS

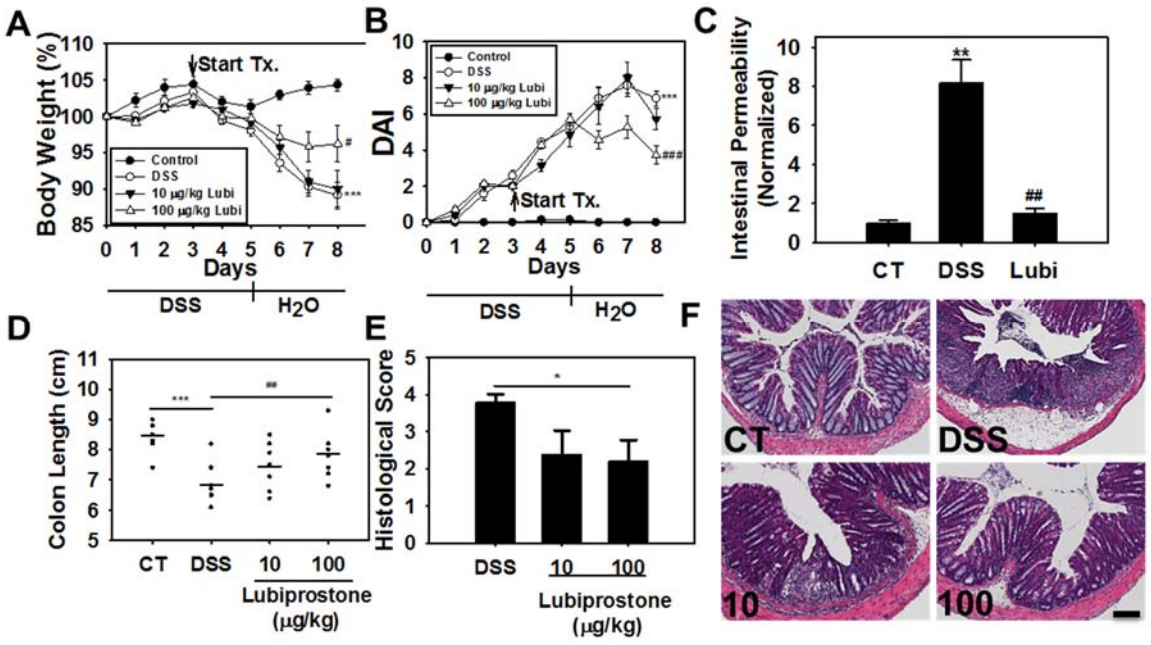


Figure 2. **Therapeutic effect of lubiprostone in DSS-induced colitis mice.** A. and B. Effect of lubiprostone on body weight (B) and disease activity index (C) in DSS-induced colitis. C. Effect of 100 μg/kg of lubiprostone on intestinal permeability in DSS-induced colitis. (n=6) D. Effect of orally treated lubiprostone on colon shortening in DSS-induced colitis (n=9). E. and F. Histologic scoring (E) and representative H&E-stained sections (F) from colonic tissue of each groups (n=5). Bar=100 μm Data are represented as means ± SEM. **p* <0.05, ***p* <0.01, and *** *p* <0.001 vs. controls (CT); # *p* <0.05, ## *p* <0.01, and ### *p* <0.001 vs. DSS without lubiprostone; one-way ANOVA

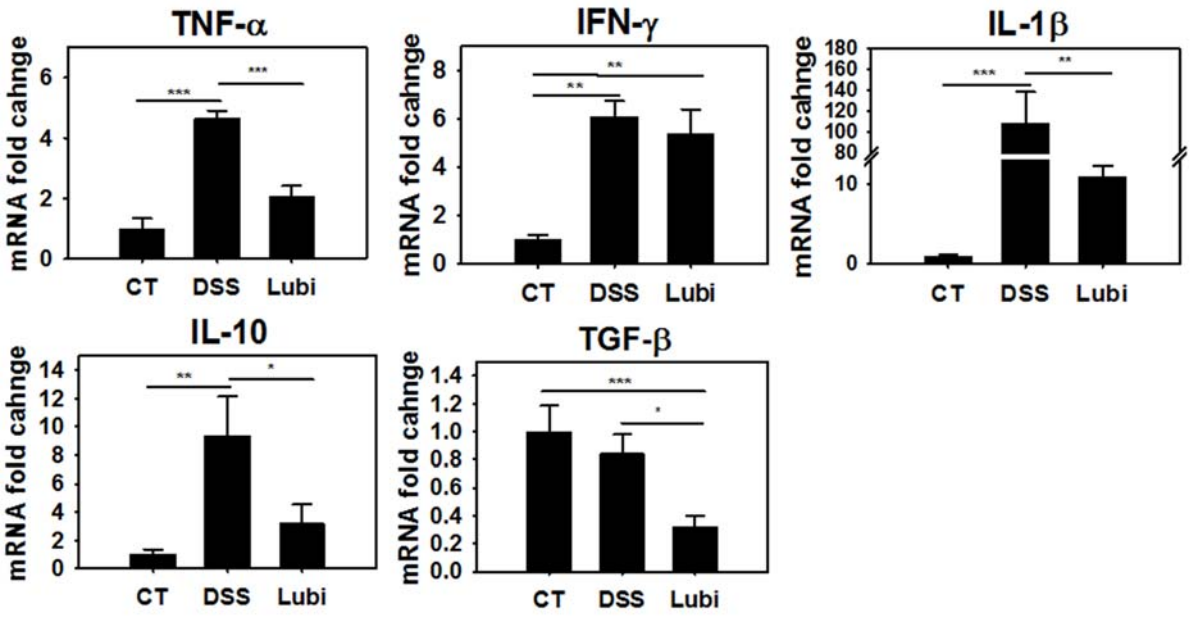
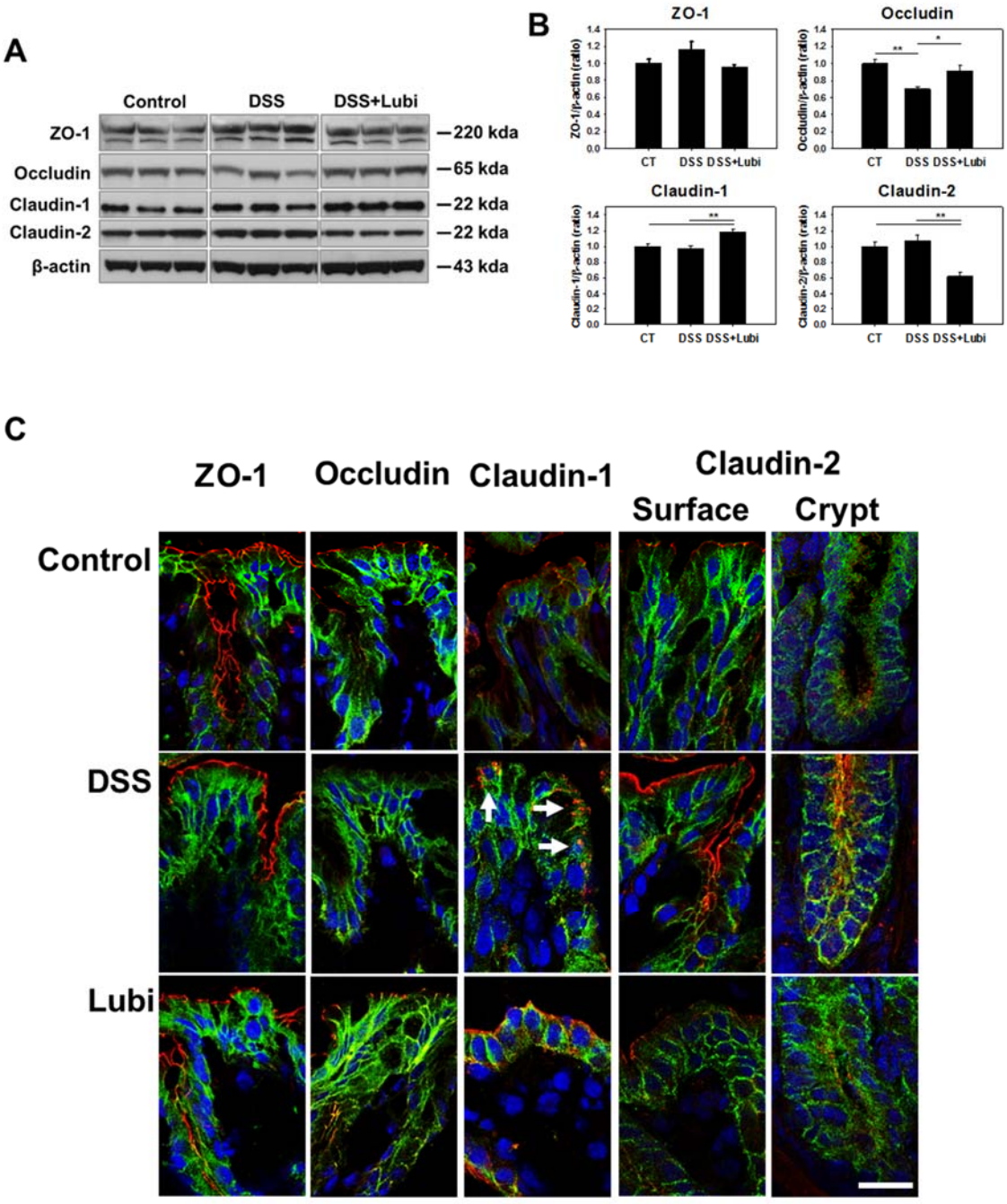


Figure 3. **Inflammatory mediators in DSS-induced colitis colonic tissues.** The real time PCR analysis of DSS colitis tissue showed significant reduced of inflammatory responses. The mRNA expression of respective cytokines was normalized to mRNA expression of β-actin. Data are represented as means ± SEM (n=5 for each group). **p* <0.05, ***p* <0.01, and *** *p* <0.001 vs. controls (CT) or DSS without lubiprostone; one-way ANOVA.

Figure 4. **Lubiprostone treatment altered expression and distribution of tight junction proteins in DSS-induced colitis.** A. Whole tissue extracts of the control, DSS, and 100 μg/kg lubiprostone mucosa were studied for expression of tight junction proteins by western blotting. Blots were loaded with equal amounts of protein per lane. B. Densitometry analysis was performed to quantify expression utilizing β–actin as loading control. Data are represented as means ± SEM. (n=4) **p* <0.05, ***p* <0.01, and *** *p* <0.001; one-way ANOVA. C. Sections of colon tissue of control, DSS, or lubiprostone (100 μg/kg) groups were immunolableled for ZO-1, Occludin, Claudin-1, and Claudin-2. Confocal microscopy images include CD326 (EpCAM) staining of basolateral surface of epithelia and TO-PRO®-3 lodide staining of nuclei. *White arrows* indicate endocytosis of claudin-1 on mucosal epithelial apical membrane. Bars =20 μm.


A
Control
DSS
DSS+Lubi
ZO-1
Occludin
Claudin-1
Claudin-2
β-actin
—220 kda
—65 kda
—22 kda
—22 kda
—43 kda
B
ZO-1
Occludin
Claudin-1
Claudin-2
ZO-1/β-actin (ratio)
Occludin/β-actin (ratio)
Claudin-1/β-actin (ratio)
Claudin-2/β-actin (ratio)
CT
DSS
DSS+Lubi
C
ZO-1
Occludin
Claudin-1
Claudin-2
Surface
Crypt
Control
DSS
Lubi

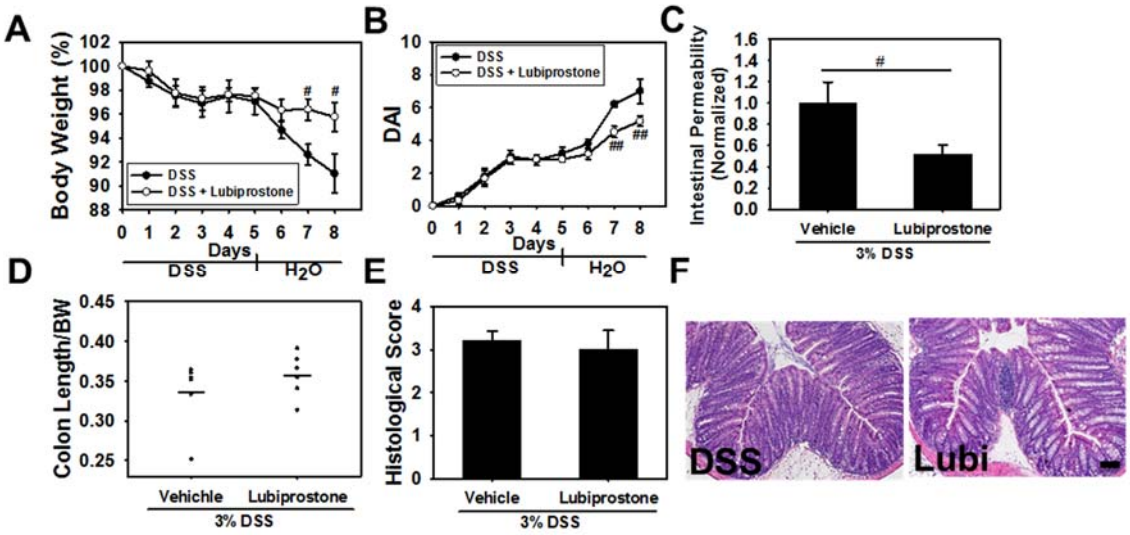
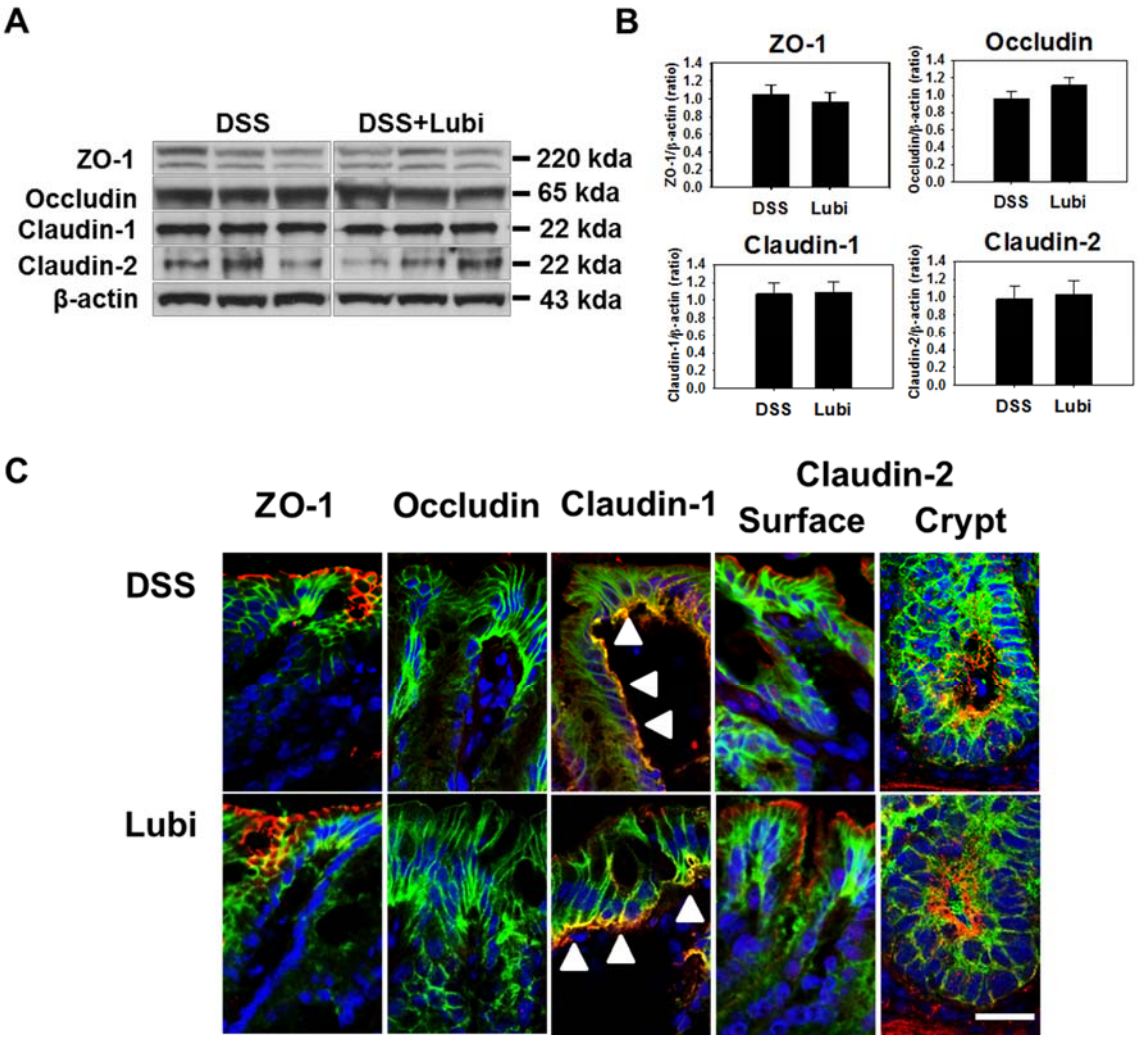


Figure 5. **Effect of high dose lubiprostone in ClC-2$^{-/-}$ mice DSS-induced colitis**. A. and B. lubiprostone reduced clinical sign of DSS colitis, demonstrated by weight-loss curve (A) and disease activity index (B). (n=5) C. Effect of lubiprostone on colon shortening in DSS-induced colitis. (n=5) D and E. Histological scoring (D) and representative H&E-stained sections (E) from colonic tissue of each groups. (n=5) Bar=100 μm #p <0.05 and ## p <0.01, vs. DSS without lubiprostone; Student's t-test

Figure 6. **Lubiprostone treatment did not alter expression of tight junction proteins in ClC-2$^{-/-}$ mice DSS-induced colitis.** A. Whole tissue extracts of the DSS and 100 μg/kg lubiprostone mucosa were studied for expression of tight junction proteins by western blotting. Blots were loaded with equal amounts of protein per lane. B. Densitometry analysis was performed to quantify expression utilizing β–actin as loading control. Data are represented as means ± SEM. (n=5) C. Sections of colon tissue of control and lubiprostone (100 μg/kg) groups were immunolableled for ZO-1, Claudin-1, Claudin-2, and Occludin. Confocal microscopy images include CD326 (EpCAM) staining of basolateral surface of epithelia and TO-PRO®-3 lodide staining of nuclei. White arrowhead indicate redistribution of claudin-1 into the basolateral membrane. Bars =20 μm.


A
DSS
DSS+Lubi
ZO-1
Occludin
Claudin-1
Claudin-2
β-actin
220 kda
65 kda
22 kda
22 kda
43 kda
B
ZO-1
Occludin
Claudin-1
Claudin-2
ZO-1/β-actin (ratio)
Occludin/β-actin (ratio)
Claudin-1/β-actin (ratio)
Claudin-2/β-actin (ratio)
DSS
Lubi
C
ZO-1
Occludin
Claudin-1
Claudin-2
Surface
Crypt
DSS
Lubi

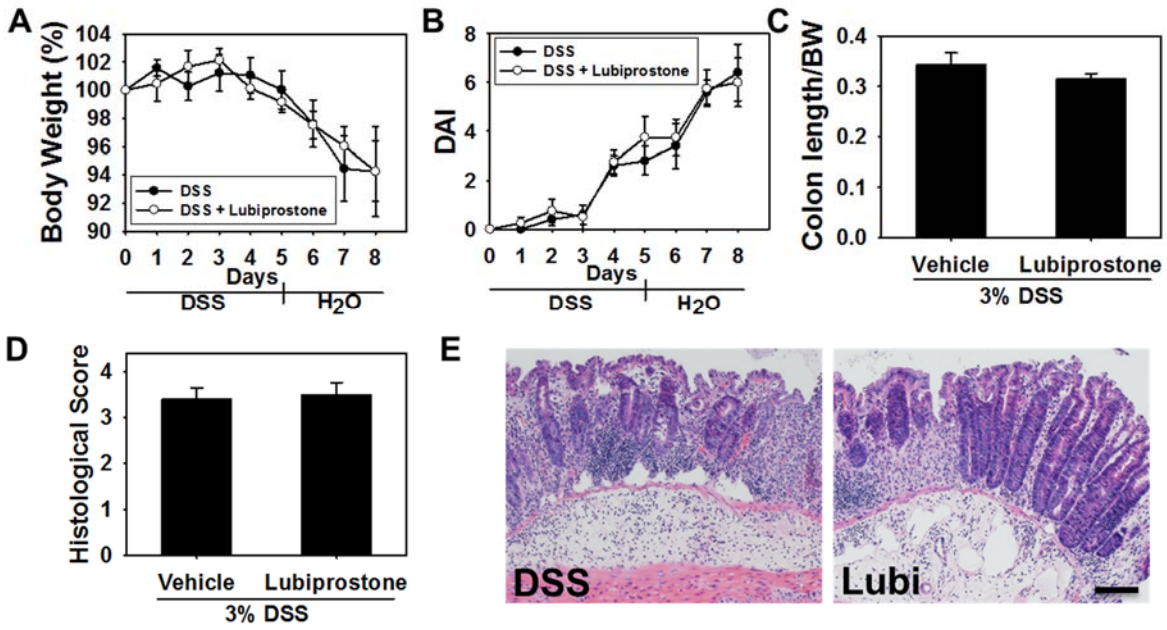


Figure 7. **Effect of low dose lubiprostone in ClC-2$^{-/-}$ mice DSS-induced colitis**. A. and B. lubiprostone reduced clinical sign of DSS colitis, demonstrated by weight-loss curve (A) and disease activity index (B). (n=5) C. Effect of lubiprostone on colon shortening in DSS-induced colitis. (n=5) D and E. Histological scoring (D) and representative H&E-stained sections (E) from colonic tissue of each groups. (n=5) Bar = 100 μm #p <0.05 and ## p <0.01, vs. DSS without lubiprostone; Student's t-test

Figure 8. Oral treatment of lubiprostone increased goblet cell differentiation in DSS-induced ClC-2$^{-/-}$ mice. A. and B. Crypt height in H&E stained colonic cross sections was measured using Image J software. C. and D. The ratio of proliferating cell per crypt was quantified using PCNA staining sections. E. Immunohistochemical staining of CA II. *Brackets* indicates highly expressed CAII in the crypts. F and G. The ratio of EC per crypts was quantified using CgA staining sections. *Arrow heads* indicate CgA positive cells. H. and I. The number of goblet cell per crypt was quantified using Alcian blue staining from colonic sections. Bar = 50 μm. Data are represented as means ± SEM (n=5 or 6 for each group). ****p* <0.001vs. DSS without lubiprostone; Student's t-test

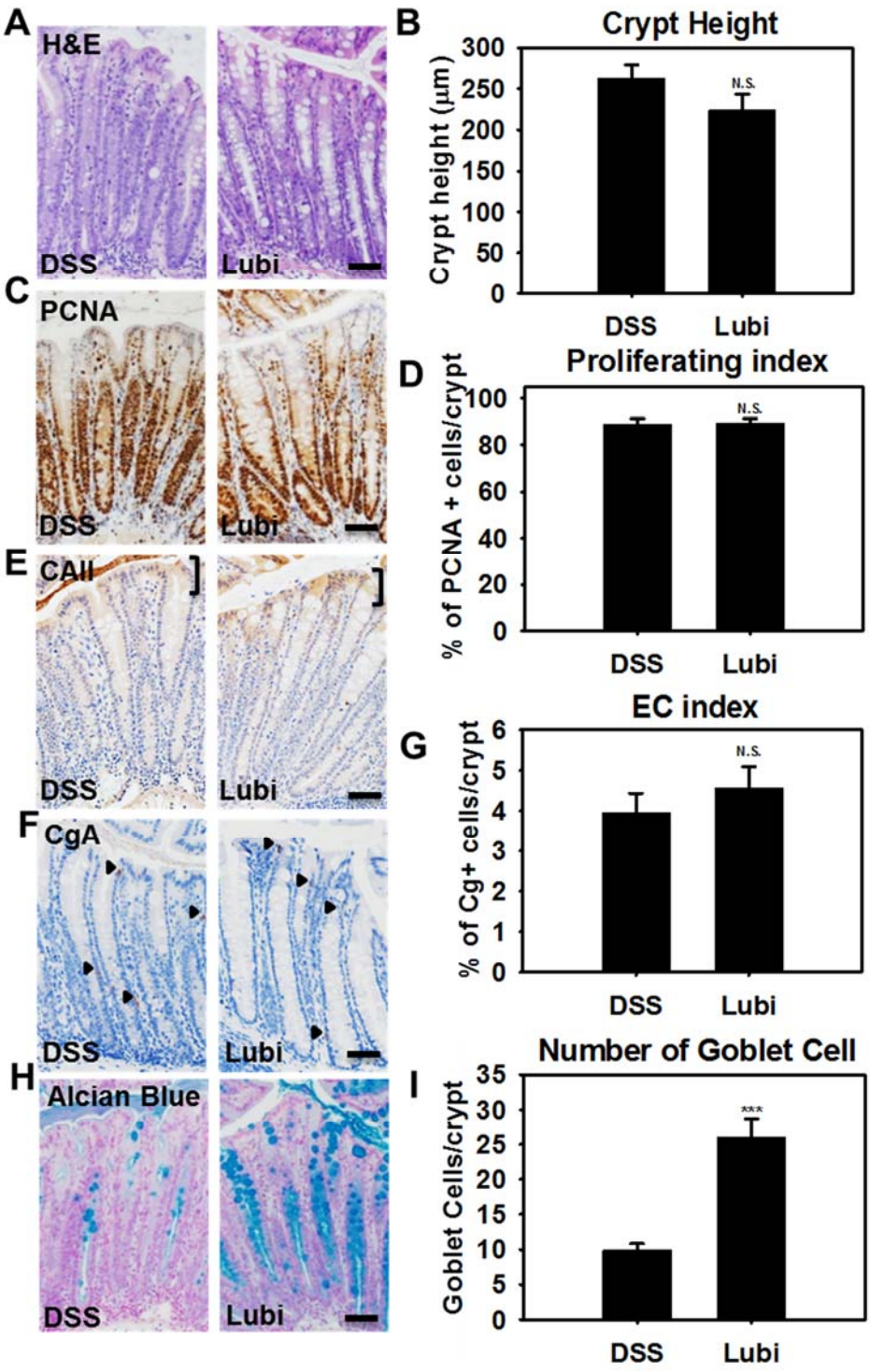
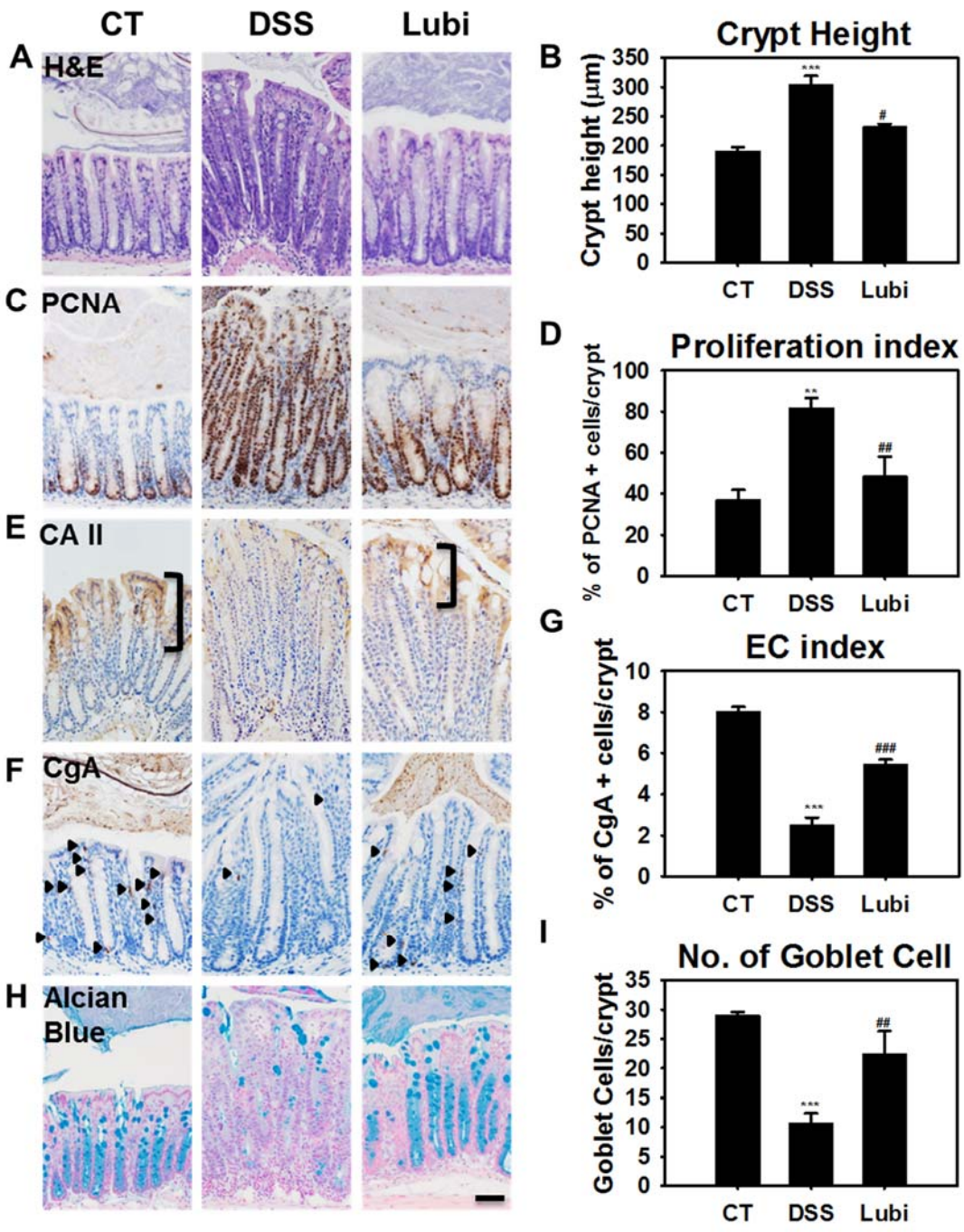

A
H&E
DSS
Lubi
B
Crypt Height
Crypt height (μm)
300
250
200
150
100
50
0
N.S.
DSS
Lubi
C
PCNA
DSS
Lubi
D
Proliferating index
% of PCNA + cells/crypt
100
80
60
40
20
0
N.S.
DSS
Lubi
E
CAII
DSS
Lubi
F
CgA
DSS
Lubi
G
EC index
% of Cg+ cells/crypt
6
5
4
3
2
1
0
N.S.
DSS
Lubi
H
Alcian Blue
DSS
Lubi
I
Number of Goblet Cell
Goblet Cells/crypt
35
30
25
20
15
10
5
0
***
DSS
Lubi

Figure 9. **Oral treatment of lubiprostone decrease cellular turnover and induced goblet cell differentiation.** A. and B. Crypt height in H&E stained colonic cross sections were measured using Image J software. C. and D. The ratio of proliferating cell per crypt was quantified using PCNA staining sections. E. Immunohistochemical staining of CA II. *Brackets* indicates highly expressed CAII in the crypts. F and G. The ratio of EC per crypts was quantified using CgA staining sections. *Arrow heads* indicate CgA positive cells. H. and I. The number of goblet cell per crypt was quantified using Alcian blue staining from colonic sections. Bar = 50 μm. Data are represented as means ± SEM (n=4 or 5 for each group). ***p* <0.01 and ****p* <0.001 vs. controls (CT); #*p* <0.05; ##*p* <0.01 and ### *p* <0.001 vs. DSS without lubiprostone; one-way ANOVA.


CT
DSS
Lubi
A
H&E
B
Crypt Height
Crypt height (μm)
350
300
250
200
150
100
50
0
***
#
CT
DSS
Lubi
C
PCNA
D
Proliferation index
% of PCNA + cells/crypt
100
80
60
40
20
0
**
##
CT
DSS
Lubi
E
CA II
G
EC index
% of CgA + cells/crypt
10
8
6
4
2
0
***
###
CT
DSS
Lubi
F
CgA
I
No. of Goblet Cell
Goblet Cells/crypt
35
30
25
20
15
10
5
0
***
##
CT
DSS
Lubi
H
Alcian Blue

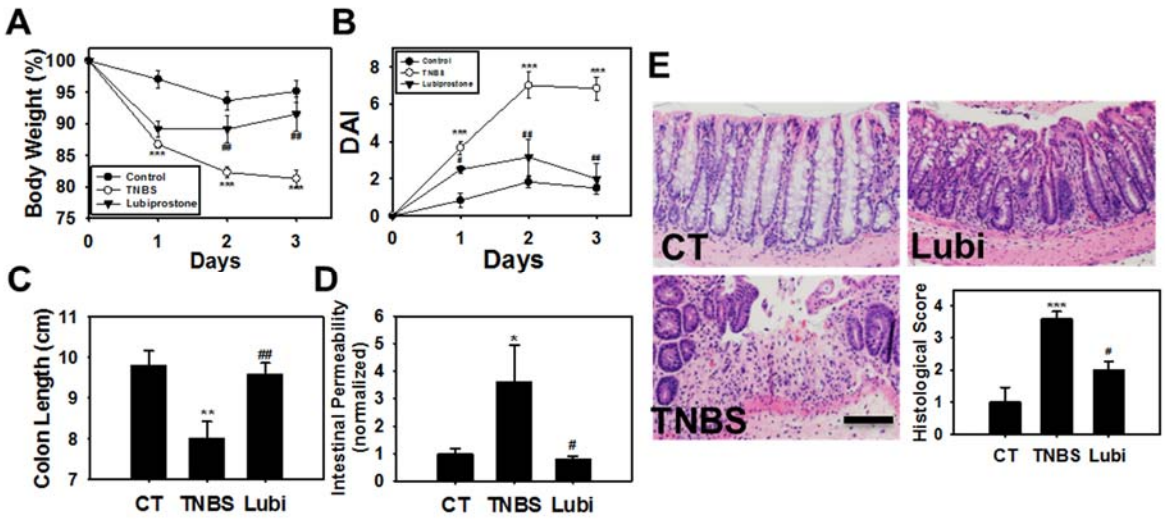


Figure 10. **Preventive effect of lubiprostone in TNBS-induced colitis.** A. and B. lubiprostone reduced clinical sign of DSS colitis, demonstrated by weight-loss curve (A) and disease activity index (B). (n=9) C. Effect of lubiprostone on colon shortening in DSS-induced colitis. (n=9) D. *In vivo* permeability was evaluated utilizing 4 kDa FITC-dextran. (n=6) E. Representative H&E-stained sections and histologic scoring from colonic tissue of each groups. (n=5) * p <0.05 and *** p <0.001 vs. controls (CT); #p <0.05 and ##p <0.01, vs. TNBS without lubiprostone; one-way ANOVA

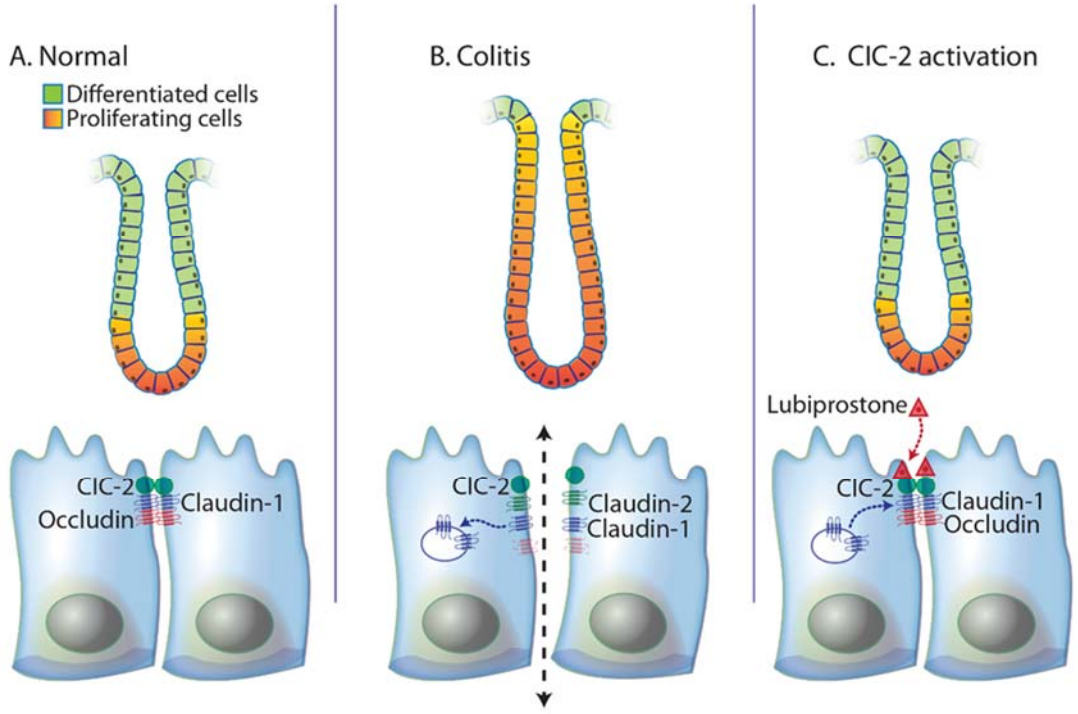


Figure 11. Pharmaceutical ClC-2 activation regulate the tight junction barrier and epithelial proliferation/differentiation. A. In differentiated intestinal mucosa, ClC-2 associated with intestinal proliferation/differentiation and contributed to the organization of tight junction proteins. B. In the colitis mucosa, redistributed and altered tight junction proteins are accompanied by increased paracellular permeability and dysregulated proliferation/differentiation. C. Lubiprostone induced ClC-2 activation protect tight junction structure and stabilize epithelial mucosa.

REFERENCES

1. Blikslager AT, Moeser AJ, Gookin JL, et al. Restoration of barrier function in injured intestinal mucosa. Physiological reviews 2007;**87**(2):545-64 doi: 10.1152/physrev.00012.2006[published Online First: Epub Date]|.
2. McGuckin MA, Eri R, Simms LA, et al. Intestinal barrier dysfunction in inflammatory bowel diseases. Inflammatory bowel diseases 2009;**15**(1):100-13 doi: 10.1002/ibd.20539[published Online First: Epub Date]|.
3. Su L, Nalle SC, Shen L, et al. TNFR2 activates MLCK-dependent tight junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis. Gastroenterology 2013;**145**(2):407-15 doi: 10.1053/j.gastro.2013.04.011[published Online First: Epub Date]|.
4. Cipriani S, Mencarelli A, Chini MG, et al. The bile acid receptor GPBAR-1 (TGR5) modulates integrity of intestinal barrier and immune response to experimental colitis. PloS one 2011;**6**(10):e25637 doi: 10.1371/journal.pone.0025637[published Online First: Epub Date]|.
5. Marchiando AM, Graham WV, Turner JR. Epithelial barriers in homeostasis and disease. Annual review of pathology 2010;**5**:119-44 doi: 10.1146/annurev.pathol.4.110807.092135[published Online First: Epub Date]|.
6. Mandel LJ, Bacallao R, Zampighi G. Uncoupling of the molecular 'fence' and paracellular 'gate' functions in epithelial tight junctions. Nature 1993;**361**(6412):552-5 doi: 10.1038/361552a0[published Online First: Epub Date]|.

7. Matter K, Aijaz S, Tsapara A, et al. Mammalian tight junctions in the regulation of epithelial differentiation and proliferation. Current opinion in cell biology 2005;**17**(5):453-8 doi: 10.1016/j.ceb.2005.08.003[published Online First: Epub Date]|.

8. Pastorelli L, De Salvo C, Mercado JR, et al. Central Role of the Gut Epithelial Barrier in the Pathogenesis of Chronic Intestinal Inflammation: Lessons Learned from Animal Models and Human Genetics. Frontiers in immunology 2013;**4**:280 doi: 10.3389/fimmu.2013.00280[published Online First: Epub Date]|.

9. Hering NA, Fromm M, Schulzke JD. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. The Journal of physiology 2012;**590**(Pt 5):1035-44 doi: 10.1113/jphysiol.2011.224568[published Online First: Epub Date]|.

10. Edelblum KL, Turner JR. The tight junction in inflammatory disease: communication breakdown. Current opinion in pharmacology 2009;**9**(6):715-20 doi: 10.1016/j.coph.2009.06.022[published Online First: Epub Date]|.

11. Poritz LS, Garver KI, Green C, et al. Loss of the tight junction protein ZO-1 in dextran sulfate sodium induced colitis. The Journal of surgical research 2007;**140**(1):12-9 doi: 10.1016/j.jss.2006.07.050[published Online First: Epub Date]|.

12. Boltin D, Perets TT, Vilkin A, et al. Mucin function in inflammatory bowel disease: an update. Journal of clinical gastroenterology 2013;**47**(2):106-11 doi: 10.1097/MCG.0b013e3182688e73[published Online First: Epub Date]|.

13. Einerhand AW, Renes IB, Makkink MK, et al. Role of mucins in inflammatory bowel disease: important lessons from experimental models. European journal of gastroenterology & hepatology 2002;**14**(7):757-65

14. Thiemann A, Grunder S, Pusch M, et al. A chloride channel widely expressed in epithelial and non-epithelial cells. Nature 1992;**356**(6364):57-60 doi: 10.1038/356057a0[published Online First: Epub Date]|.

15. Nighot P, Young K, Nighot M, et al. Chloride Channel ClC-2 is a Key Factor in the Development of DSS-induced Murine Colitis. Inflammatory bowel diseases 2013 doi: 10.1097/MIB.0b013e3182a82ae9[published Online First: Epub Date]|.

16. Nighot PK, Blikslager AT. Chloride channel ClC-2 modulates tight junction barrier function via intracellular trafficking of occludin. Am J Physiol Cell Physiol 2012;**302**(1):C178-87 doi: ajpcell.00072.2011 [pii]

10.1152/ajpcell.00072.2011[published Online First: Epub Date]|.

17. Nighot PK, Blikslager AT. ClC-2 regulates mucosal barrier function associated with structural changes to the villus and epithelial tight junction. American journal of physiology. Gastrointestinal and liver physiology 2010;**299**(2):G449-56 doi: 10.1152/ajpgi.00520.2009[published Online First: Epub Date]|.

18. Nighot PK, Moeser AJ, Ryan KA, et al. ClC-2 is required for rapid restoration of epithelial tight junctions in ischemic-injured murine jejunum. Experimental cell research 2009;**315**(1):110-8 doi: 10.1016/j.yexcr.2008.10.001[published Online First: Epub Date]|.

19. Moeser AJ, Nighot PK, Engelke KJ, et al. Recovery of mucosal barrier function in ischemic porcine ileum and colon is stimulated by a novel agonist of the ClC-2 chloride channel, lubiprostone. American journal of physiology. Gastrointestinal and liver physiology 2007;**292**(2):G647-56 doi: 10.1152/ajpgi.00183.2006[published Online First: Epub Date]|.

20. Moeser AJ, Haskell MM, Shifflett DE, et al. ClC-2 chloride secretion mediates prostaglandin-induced recovery of barrier function in ischemia-injured porcine ileum. Gastroenterology 2004;**127**(3):802-15

21. Gyomorey K, Yeger H, Ackerley C, et al. Expression of the chloride channel ClC-2 in the murine small intestine epithelium. Am J Physiol Cell Physiol 2000;**279**(6):C1787-94

22. Kirk KL. Chloride channels and tight junctions. Focus on "Expression of the chloride channel ClC-2 in the murine small intestine epithelium". Am J Physiol Cell Physiol 2000;**279**(6):C1675-6

23. Cryer B, Katz S, Vallejo R, et al. A Randomized Study of Lubiprostone for Opioid-Induced Constipation in Patients with Chronic Noncancer Pain. Pain medicine 2014 doi: 10.1111/pme.12437[published Online First: Epub Date]|.

24. Ueno R, Osama H, Habe T, et al. Oral SPI-0211 increases intestinal fluid secretion and chloride concentration without altering serum electrolyte levels. Gastroenterology 2004;**126**(4):A298-A98

25. Nehrke K, Arreola J, Nguyen HV, et al. Loss of hyperpolarization-activated Cl(-) current in salivary acinar cells from Clcn2 knockout mice. J Biol Chem 2002;**277**(26):23604-11 doi: 10.1074/jbc.M202900200

M202900200 [pii][published Online First: Epub Date]|.

26. Matos I, Bento AF, Marcon R, et al. Preventive and therapeutic oral administration of the pentacyclic triterpene alpha,beta-amyrin ameliorates dextran sulfate sodium-induced colitis in mice: the relevance of cannabinoid system. Molecular immunology 2013;**54**(3-4):482-92 doi: 10.1016/j.molimm.2013.01.018[published Online First: Epub Date]|.

27. Wirtz S, Neufert C, Weigmann B, et al. Chemically induced mouse models of intestinal inflammation. Nature protocols 2007;**2**(3):541-6 doi: 10.1038/nprot.2007.41[published Online First: Epub Date]|.

28. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2008;**22**(3):659-61 doi: 10.1096/fj.07-9574LSF[published Online First: Epub Date]|.

29. Amasheh S, Milatz S, Krug SM, et al. Tight junction proteins as channel formers and barrier builders. Ann N Y Acad Sci 2009;**1165**:211-9 doi: 10.1111/j.1749-6632.2009.04439.x

NYAS04439 [pii][published Online First: Epub Date]|.

30. Cuppoletti J, Malinowska DH, Tewari KP, et al. SPI-0211 activates T84 cell chloride transport and recombinant human ClC-2 chloride currents. Am J Physiol Cell Physiol

2004;**287**(5):C1173-83 doi: 10.1152/ajpcell.00528.2003[published Online First: Epub Date]|.

31. Schiffhauer ES, Vij N, Kovbasnjuk O, et al. Dual activation of CFTR and CLCN2 by lubiprostone in murine nasal epithelia. American journal of physiology. Lung cellular and molecular physiology 2013;**304**(5):L324-31 doi: 10.1152/ajplung.00277.2012[published Online First: Epub Date]|.

32. Norimatsu Y, Moran AR, MacDonald KD. Lubiprostone activates CFTR, but not ClC-2, via the prostaglandin receptor (EP(4)). Biochemical and biophysical research communications 2012;**426**(3):374-9 doi: 10.1016/j.bbrc.2012.08.097[published Online First: Epub Date]|.

33. Jakab RL, Collaco AM, Ameen NA. Lubiprostone targets prostanoid signaling and promotes ion transporter trafficking, mucus exocytosis, and contractility. Digestive diseases and sciences 2012;**57**(11):2826-45 doi: 10.1007/s10620-012-2352-8[published Online First: Epub Date]|.

34. Ao M, Venkatasubramanian J, Boonkaewwan C, et al. Lubiprostone activates Cl- secretion via cAMP signaling and increases membrane CFTR in the human colon carcinoma cell line, T84. Digestive diseases and sciences 2011;**56**(2):339-51 doi: 10.1007/s10620-010-1495-8[published Online First: Epub Date]|.

35. Renes IB, Verburg M, Van Nispen DJ, et al. Epithelial proliferation, cell death, and gene expression in experimental colitis: alterations in carbonic anhydrase I, mucin MUC2, and trefoil factor 3 expression. International journal of colorectal disease

2002;**17**(5):317-26 doi: 10.1007/s00384-002-0409-4[published Online First: Epub Date]|.

36. Pena-Munzenmayer G, Catalan M, Cornejo I, et al. Basolateral localization of native ClC-2 chloride channels in absorptive intestinal epithelial cells and basolateral sorting encoded by a CBS-2 domain di-leucine motif. Journal of cell science 2005;**118**(Pt 18):4243-52 doi: 10.1242/jcs.02525[published Online First: Epub Date]|.
37. Catalan M, Niemeyer MI, Cid LP, et al. Basolateral ClC-2 chloride channels in surface colon epithelium: regulation by a direct effect of intracellular chloride. Gastroenterology 2004;**126**(4):1104-14 doi: S0016508504000307 [pii][published Online First: Epub Date]|.
38. Cuppoletti J, Blikslager AT, Chakrabarti J, et al. Contrasting effects of linaclotide and lubiprostone on restitution of epithelial cell barrier properties and cellular homeostasis after exposure to cell stressors. BMC pharmacology 2012;**12**(1):3 doi: 10.1186/1471-2210-12-3[published Online First: Epub Date]|.
39. Boismenu R, Chen Y. Insights from mouse models of colitis. Journal of leukocyte biology 2000;**67**(3):267-78
40. Kucharzik T, Walsh SV, Chen J, et al. Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. The American journal of pathology 2001;**159**(6):2001-9 doi: 10.1016/S0002-9440(10)63051-9[published Online First: Epub Date]|.
41. Weber CR, Nalle SC, Tretiakova M, et al. Claudin-1 and claudin-2 expression is elevated in inflammatory bowel disease and may contribute to early neoplastic transformation.

Lab Invest 2008;**88**(10):1110-20 doi: 10.1038/labinvest.2008.78[published Online First: Epub Date]|.

42. Lameris AL, Huybers S, Kaukinen K, et al. Expression profiling of claudins in the human gastrointestinal tract in health and during inflammatory bowel disease. Scandinavian journal of gastroenterology 2013;**48**(1):58-69 doi: 10.3109/00365521.2012.741616[published Online First: Epub Date]|.
43. Turner JR. Intestinal mucosal barrier function in health and disease. Nature reviews. Immunology 2009;**9**(11):799-809 doi: 10.1038/nri2653[published Online First: Epub Date]|.
44. Heller F, Florian P, Bojarski C, et al. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology 2005;**129**(2):550-64 doi: DOI 10.1053/j.gastro.2005.05.002[published Online First: Epub Date]|.
45. Tsukita S, Yamazaki Y, Katsuno T, et al. Tight junction-based epithelial microenvironment and cell proliferation. Oncogene 2008;**27**(55):6930-8 doi: 10.1038/onc.2008.344[published Online First: Epub Date]|.
46. Tamura A, Kitano Y, Hata M, et al. Megaintestine in claudin-15-deficient mice. Gastroenterology 2008;**134**(2):523-34 doi: 10.1053/j.gastro.2007.11.040[published Online First: Epub Date]|.
47. Dhawan P, Singh AB, Deane NG, et al. Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. The Journal of clinical investigation 2005;**115**(7):1765-76 doi: 10.1172/JCI24543[published Online First: Epub Date]|.

CHAPTER 4

MYOSIN LIGHT CHAIN KINASE MEDIATES INTESTINAL BARRIER DYSFUNCTION VIA OCCLUDIN ENDOCYTOSIS DURING HYPOXIA/REOXYGENATION INJURY.

ABSTRACTS

Intestinal hypoxia/reoxygenation injury induces loss of barrier function followed by epithelial repair. The tight junction (TJ) has recently been characterized as key factor for intestinal barrier properties. Myosin light chain (MLC) phosphorylation mediated by MLC kinase (MLCK) is essential to the regulation of the tight junction barrier. We hypothesized that hypoxia/reoxygenation (HR) injury would disrupt the TJ barrier function via MLC phosphorylation. Caco-2BBe1 confluent monolayers were subjected to hypoxia for 2 hours followed by recovery in 21% O_2, after which barrier function was determined by measuring flux of 4 kDa FITC-dextran. Expression and distribution of TJ components were studied by immunoblotting, real time-PCR and immunofluorescence microscope analysis. Expression of MLCK, phosphorylated MLC, and total MLC in the human intestinal cell was assessed by western blot and real time-PCR. Following hypoxic injury, there was an increase in the paracellular permeability as determined by dextran fluxes compared to control cells. Permeability recovered with significant reduction in fluxes at 4 h during reoxygenation. Expression of the TJ protein occludin was increased after hypoxic injury. Occludin was internalized during H/R injury and re-localized to its normal distribution after 4 h recovery. MLC phosphorylation was significantly increased without elevation of MLCK expression. Treatment with the MLCK specific inhibitor peptide 18 attenuated the increased epithelial monolayer permeability and occludin endocytosis caused by H/R injury. These data suggest that the MLC phosphorylation-induced occludin endocytosis mediates intestinal epithelial barrier dysfunction during the H/R injury. Our results also indicates that MLCK-dependent

occludin regulation may be a critical target for the therapeutic treatment of clinical ischemic/reperfusion injury.

INTRODUCTION

Intestinal ischemia/reperfusion (I/R) injury is usually secondary to diverse conditions, such as mesenteric artery embolism, small bowel transplantation, cardiopulmonary bypass, abdominal aortic aneurysm surgery, traumatic or hemorrhagic shock, and inflammatory bowel disease. I/R injury may initiate mucosal epithelial cell damage, breakdown of basement membrane integrity and barrier function, and may cause systemic inflammatory response syndrome (SIRS). [1 2] The intestinal mucosal barrier is a physical and metabolic barrier against toxins and pathogens in the lumen and its mechanisms are very complicated. [3] However, the molecular basis of barrier function in I/R injury in the intestine as well as the cellular mechanisms are currently unknown.

Tight junctions between intestinal epithelial cells are largely attributable for defining the characteristics of the intestinal mucosal barrier. The tight junction strands consist of transmembrane proteins such as occludin and claudins as well as peripheral membrane proteins, including the zona occludens proteins, ZO-1-3, which are bound to the perijunctional actomyosin ring by direct and indirect protein-protein interaction. [4-7] Disrupted intestinal tight junctions have been observed in various digestive diseases such as stress, burn injury, I/R injury, and inflammatory bowel disease. [8-13] Thus, disruption of tight junction proteins is believed to be the key limiting factor of the intestinal mucosal paracellular permeability. Our previous studies have demonstrated that restoration of tight

junction proteins in ischemic-injured porcine and mouse small intestines are associated with recovery of barrier function. [14-19]

The perijunctional actomyosin ring contraction due to myosin light chain (MLC) phosphorylation, which predominantly depends on myosin light chain kinase (MLCK) activation, has been demonstrated to be necessary for tight junction opening. MLCK-induced MLC phosphorylation leads to perijunctional actomyosin contraction and breakdown of the tight junction proteins ZO-1 occludin. [20 21] Thus, MLCK-dependent tight junction remodeling appears to be critical to the pathophysiologically disrupted intestinal barrier.

In this study, we hypothesized that MLCK-dependent reorganization of tight junction proteins might be an essential component of epithelial barrier maintenance during *in vitro* hypoxia/reoxygenation (H/R) injury. To investigate this, we examined intestinal barrier function at various time points in a H/R injury model. H/R injury elevated the paracellular permeability of FITC-dextran via occludin internalization with increased MLC phosphorylation. Inhibition of MLCK with peptide 18 significantly reduced paracellular permeability and occludin internalization. Our findings indicate that MLCK-dependent MLC phosphorylation mediates intestinal epithelial barrier dysfunction during H/R injury.

MATERIAL AND METHODS

Cell Culture

A human intestine C2Bbe1 (Caco-2BBe1) cell line derived from Caco-2 cells was obtained from ATCC (Manassas, VA) and grown in standard DMEM with 10% FBS and 0.01 mg/ml human transferrin (Invitrogen, Carlsbad, CA). Cells were maintained in a humidified 37°C, 5% CO_2 incubator. To prepare Caco-2BBe1 monolayers, cells were grown

on cell culture-treated surfaces or 12 mm 0.4 μm pore-sized permeable supports (Corning, Tewksbury, MA).

Hypoxia/Reoxygenation Experiments

To generate hypoxic injury, Caco-2BBe1 cells were placed in a modular incubator chamber (Billups-Rothenberg Inc., San Diego, CA) and flushed with 95% N_2/5% CO_2 for 5 minutes. The modular chamber was then sealed air-tight and placed in an incubator for 2 hours. After 2 hours, the cells were removed from the modular incubator chamber and placed in a 21% O_2 normal environment. The cells were then incubated under normal environmental conditions for 1, 2, and 4 hour incubation periods.

Measurement of Fluorescent-Dextran Flux

Cells on permeable support were used in this experiment only after a transepithelial electrical resistance (TER) value greater than 400 $\Omega \cdot cm^2$ was measured Paracellular permeability was assessed by apical to basal flux of fluorescein-conjugated dextran (FD-4; Sigma-Aldrich, St. Louis, MO). 1 mg/mL of FD-4 was added to the apical chambers after hypoxic injury. Triplet 100 μL basolateral media was sampled and assayed for fluorescence using a fluorescent spectrophotometer (excitation, 488 nm; emission, 530 nm). These measurements were used to calculate the solute permeability coefficient of the monolayer. [22]

Gel Electrophoresis and Western Blotting

The cell monolayers were washed with PBS and scraped in M-PER mammalian protein extraction reagent including a protease inhibitor cocktail consisting of 1 mM NaF and 1 mM $NaVO_4$ (Thermo Scientific, Rockford, IL). The proteins were extracted according to

the manufacturer's instructions. Protein analysis of extract aliquots was performed using BCA protein assay kit (Thermo Scientific, Rockford, IL). Tissue extracts, with amounts equalized by protein concentration, were mixed with XT sample buffer and a reducing agent (both Bio-Rad Laboratories Inc., Hercules, CA) and boiled for 5 min. Lysates were loaded on a 4-10% SDS polyacrylamide gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a PVDF membrane (Bio-Rad Laboratories Inc., Hercules, CA) by using an electroblotting transfer apparatus. Membranes were blocked in Tris-buffered saline plus 0.1% Tween20 (TBST) and 5% BSA or skim milk and then incubated overnight with primary antibodies at 4°C. The primary antibodies used were occludin (Invitrogen, Carlsbad, CA), pMLC-2, MLC-2 (Cell signaling, Danvers, MA), and HIF-1α (Abcam Inc., Cambridge, MA). After being washed in TBST, membranes were incubated with horseradish peroxidase conjugated secondary antibodies and developed for protein visualization with chemiluminescence (Thermo Scientific, Rockford, IL).

Immunofluorescence Microscopy Analysis

The cells grown on permeable supports were fixed with absolute methanol and stored at -80°C until used. The cells were thawed, rinsed in PBS, blocked with goat normal serum, and incubated overnight at 4°C with primary antibodies. The cells were washed thoroughly and incubated in secondary antibodies conjugated with fluorescent dyes and nuclear stain. Following rinsing in PBS, the cells were mounted on fluorescent mounting media (DakoCytomation, Via Real Carpinteria, CA) and examined with an Olympus IX83 Inverted Motorized Microscope.

Effects of MLCK inhibitor.

Peptide 18 (400 μM, Calbiochem, Billerica, MA), a membrane permeant nonapeptide inhibitor of MLCK, was added to apical side 30 minute before hypoxic injury. The permeability of FD-4 and Immunofluorescence microscopy analysis was performed as described above.

Statistical Analysis

Data are reported as mean ± standard error. Differences between groups tested with one-way ANOVA with LSD post-hot test (IBM SPSS Statistics 21). Where appropriate, the difference between two groups were tested with a student t-test (Excel, Microsoft 2013).

RESULTS

H/R injury increased paracellular permeability.

Permeability of the FITC-dextran on the Caco-2BBe1 monolayer was monitored during the H/R injury as a measurement of the epithelial barrier (Fig. 1A). Hypoxic injury was confirmed by significant elevation of HIF-1α stabilization using western blot analysis (Fig. 1B). The permeability of the FITC-dextran on Caco-2BBe1 monolayers was significantly increased at 1 hour after hypoxic injury and reduced at 4 hours after hypoxic injury. (Fig. 1C) These results suggest that the H/R injury increases paracellular permeability on Caco-2BBe1 monolayer.

H/R Injury alters the expression of the TJ proteins and mRNAs.

Tight junction proteins were detected by western blot and real time PCR to investigate the mechanism of H/R injury-induced intestinal barrier dysfunction. The results showed that protein levels of ZO-1, occludin, and claudin-4 were significantly increased in

Caco-2BBe1 cells after hypoxic injury (Fig. 2A and B). However, the mRNA level of only occludin was significantly increased after hypoxic injury (Fig. 2C). Expression of other tight junction proteins or mRNA including claudin-1, -2, and -7 did not change significantly. We conclude that H/R injury had effect on the expression of tight junction proteins and mRNAs.

Occludin is disrupted and recovered during H/R Injury.

Next, we determined whether H/R injury affected the distribution of tight junction proteins in Caco-2BBe1 monolayers. To investigate tight junction protein endocytosis, mucosal extracts were fractionated into membrane and cytosol components. We used a standard loading control protein β-actin, which was expressed in equal proportions in the membrane and cytosol fractions. In the normal monolayer (-2h), Transmembrane tight junction proteins occludin and claudin-4 were mainly expressed in the membrane fraction and the cytoplasmic plaque protein ZO-1 was predominantly expressed in the cytosol fraction. However, the expression of occludin in the membrane fraction was significantly reduced after hypoxic injury and then recovered during the reoxygenation (Fig. 3).

To get more evidence about morphological redistribution of tight junction proteins, mucosal monolayers were evaluated using immunofluorescence microscope analysis. As shown in control Caco-2BBe1 monolayers, the tight junction proteins occludin were respectively localized to the intercellular tight junctions, along the edge of the cells. However, H/R injury induced pronounced reorganization of occludin such that the distribution profiles became irregular and discontinuous. In addition, the occludin was partially internalized into cytoplasmic vesicles (Fig. 4). However, immunolocalization of other tight junction proteins ZO-1, claudin-4, and claudin-7 did not change (data not shown).

Taken together, these results suggest that the epithelial barrier dysfunction during H/R injury is induced by disruption and endocytosis of occludin.

H/R injury elevates MLC-2 phosphorylation without increased MLCK expression

In several studies, occludin endocytosis is induced by increased long MLCK expression and activity [23 24]. MLCK activity was evaluated by measuring MLC-2 phosphorylation and MLCK expression was determined by measuring the mRNA level of MLCK. MLC-2 phosphorylation was significantly increased without markedly changing total MLC expression after hypoxic injury and then was reduced during the reoxygenation period (Fig 5A and B). However, the mRNA expression of MLCK was not changed during H/R injury (Fig. 5C). Thus, these results indicate that the hypoxic injury increased MLC-2 phosphorylation without increased MCLK expression.

MLCK inhibitor reduces paracellular barrier dysfunction via preventing occludin endocytosis induced by H/R injury

The data presented here suggest that increased paracellular permeability is triggered by MLCK activation-induced occludin endocytosis during hypoxia/reoxygenation injury. Thus, we hypothesized that the MLCK inhibitor reduces paracellular permeability by preventing occludin endocytosis. To test this hypothesis, we added Peptide 18 to the apical side of Caco-2BBe1 monolayer before hypoxic injury. The inhibition of MLCK was confirmed by measuring phosphorylation of MLC-2 and total MLC-2 during H/R injury. Increased phosphorylation after hypoxic injury was reduced in the monolayer treated with Peptide 18 (Fig. 6A). Monolayers treated with Peptide 18 had significantly reduced increased permeability of FD-4 during the H/R injury (Fig. 6B). Inhibition of MLCK during the H/R

injury in Caco-2BBe1 monolayer also reduced occludin endocytosis (Fig. 6C). Thus, these data suggests that MLCK inhibition prevents paracellular barrier dysfunction by preventing occludin endocytosis during H/R injury.

DISCUSSION

In this study, it is demonstrated that H/R injury causes increased paracellular permeability, and that the increased permeability induced by H/R injury is accompanied by redistribution of the tight junction protein occludin as well as upregulation of MLC phosphorylation. Our present study also reveals that MLCK inhibition with the specific inhibitor Peptide 18 attenuates the H/R injury-induced paracellular barrier disruption *in vitro*. These findings provide a new insight into the mechanisms involved in the intestinal barrier breakdown caused by H/R injury.

It has been well documented that several gastrointestinal diseases, such as inflammatory bowel disease, celiac disease, irritable bowel syndrome, and ischemic disease cause the disruption of intestinal epithelial barrier function. Increased intestinal permeability results in leakage of bacteria, microbial products or other antigens from the intestinal lumen into the lamina propria to initiate or exacerbate an inflammatory response. [18 25] Here, we demonstrate that intestinal epithelial permeability to 4 kDa FITC-dextran increased during H/R injury. Paracellular permeability across intestinal epithelium is in large part regulated by the tight junctions. In addition, several studies have demonstrated that occludin endocytosis is a common feature of several intestinal diseases and is closely associated with intestinal barrier dysfunction. [26 27] We have previously shown that occludin was internalized at 1.5 hours after ischemic injury and recovered at the 3-hour after ischemic recovery in mice

model. [15] Here, our results from tight junction analysis using western blotting and IF indicate that internalization of the tight junction transmembrane protein occludin elevates paracellular permeability during *in vitro* human intestinal H/R injury.

The mechanism of MLC-2 phosphorylation by upregulating MLCK protein expression is well characterized. Previous studies have shown that upregulating MLCK protein expression triggers occludin internalization in the intestinal epithelium. [27-29] However, our results showed that the phosphorylation of MLC-2 increased after hypoxic injury without change of MLCK expression. Treatment with Peptide 18, MLCK specific inhibitor, reduced paracellular permeability via inhibiting MLC-2 phosphorylation. MLCK is activated by Ca^{2+}/calmodulin in response to an increase in intracellular calcium. H/R stimulates intracellular calcium release from an intracellular pool that includes the endoplasmic reticulum. [30] Increased MLC-2 phosphorylation in our study may be caused by increased intracellular calcium by hypoxic injury.

The increased paracellular permeability of 4 kDa FITC-dextran was recovered 4 hours after hypoxic injury. This results indicates that there are some recovery mechanisms involved during reoxygenation. A number of studies have revealed that HIF-1α stimulates a barrier protective program in the intestine by increased nonclassical regulators of barrier function including mucin, P-glycoprotein, ecto-5'-nucleotidase, CD73, and adenosine A2B receptor. [31-33] However, we demonstrate that total protein and mRNA expression of the classic regulator occludin was significantly elevated during the recovery. In addition, we also showed that disrupted occludin localization was redistributed to the membrane at the 4 hour recovery time point. Thus, these results indicate that increased expression and redistribution

of occludin related with barrier protective program during H/R injury.

In conclusion, our present data demonstrate that the MLCK-dependent occludin internalization results in intestinal barrier dysfunction in H/R injury. To the best of our knowledge, this is the first experimental study providing evidence to show the role of occludin regulated by MLCK in intestinal barrier disruption and recovery during H/R. It is suggested that MLCK-dependent MLC phosphorylation may be a critical target for the therapeutic treatment of intestinal ischemic/reperfusion injury.

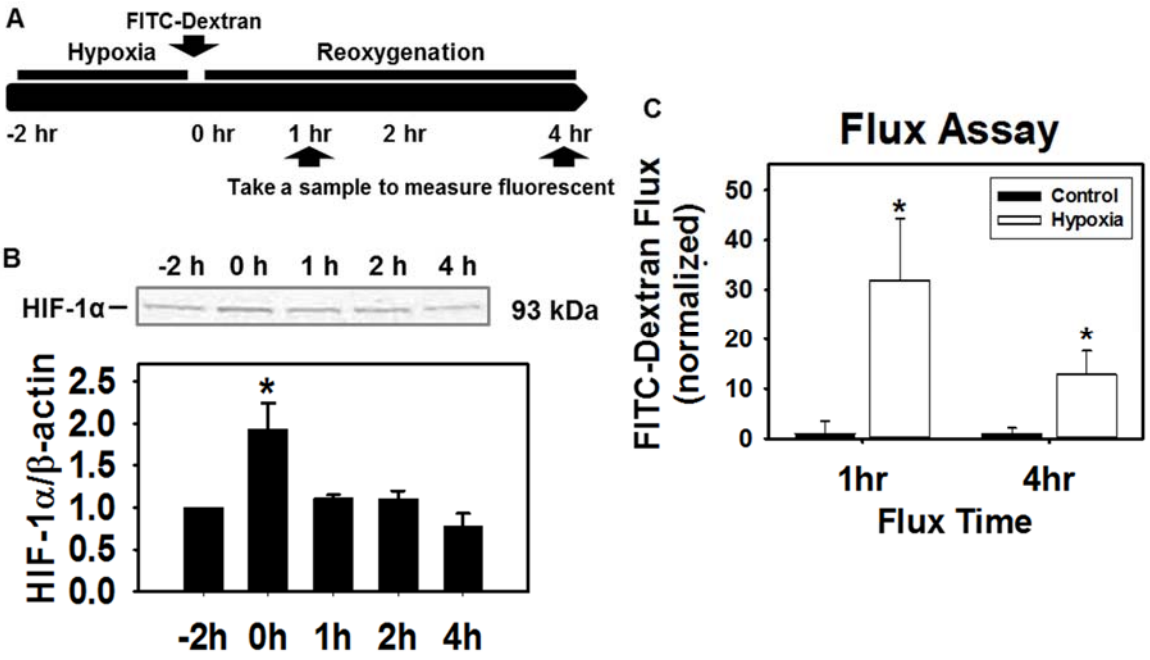
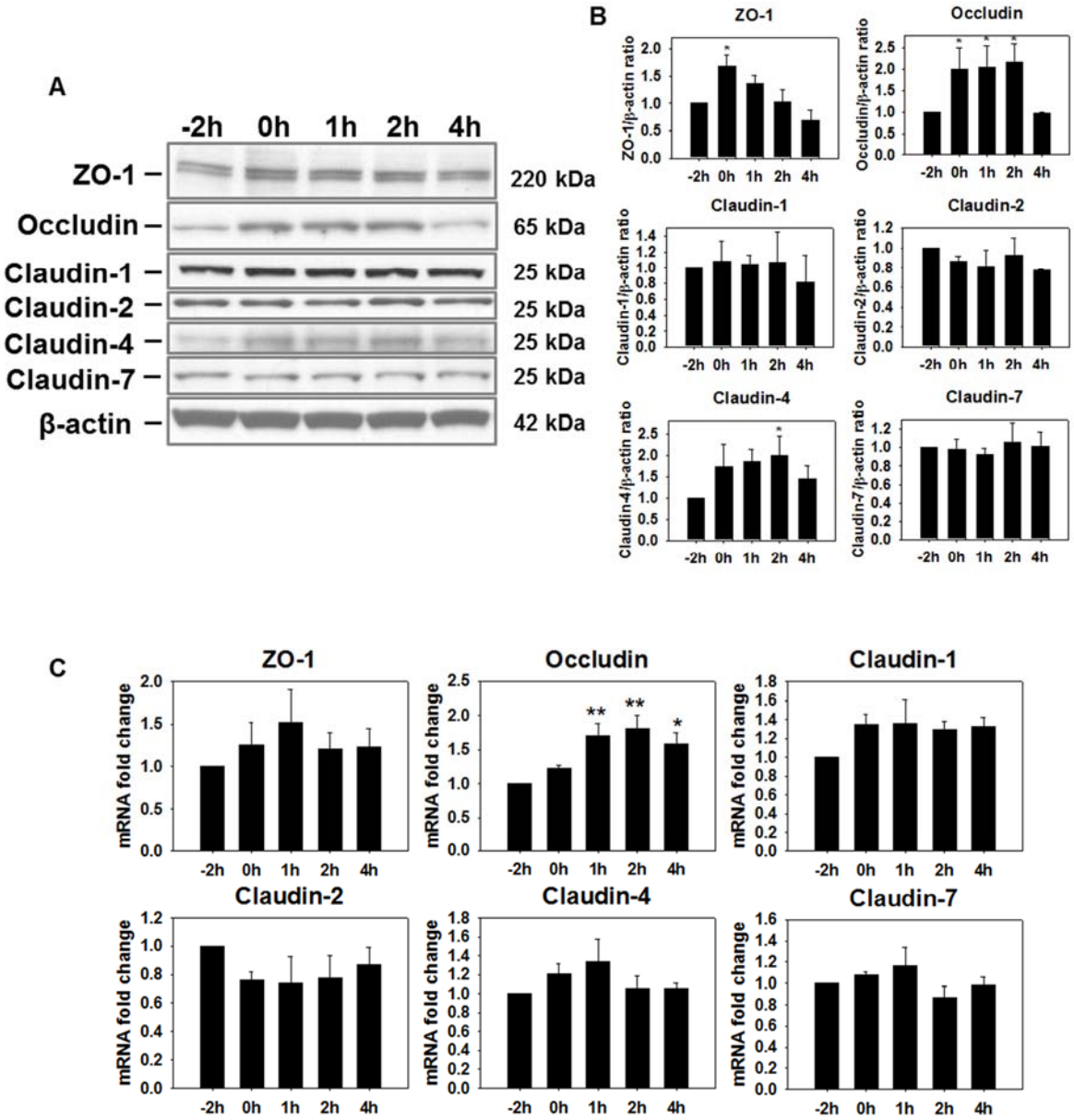


Figure 1. Evaluation of paracellular permeability during H/R injury in Caco-2BBe1 cell. (A) Schematic drawing showing the schedule of flux assay in sublethal H/R injury. (B) The hypoxic injury was confirmed by measurement of expression of HIF-1α using western blot analysis. Data are mean ± SEM (n=3 experiment). **p*<0.05, versus -2 h (C) The apical-to-basal paracellular fluxes of 4 kDa FITC-dextran were performed in hypoxia or reoxygenation period. Data are expressed as permeability coefficient. Data are mean ± SEM (n=5 at each time point).). **p*<0.05, versus control cell line at each time point.

Figure 2. Expression of TJ proteins and mRNAs in H/R injury of Caco-2BBe1 monolayer. (A) Western blot analysis of total cell lysate in hypoxia/reoxygenation injury. The TJ proteins ZO-1, occludin, and claudin-4 were increased expression after hypoxic injury. (B) Semi-quantitative analysis was performed with Image-Pro Plus software. Expression of occludin was significantly increased after hypoxic injury. (C) Real time-PCR analysis of total cell lysate during H/R injury. The TJ mRNA level of occludin was increased after hypoxic injury. Data are mean ± SEM (n=3-4 at each time point).). **p*<0.05, versus -2h.


A
-2h
0h
1h
2h
4h
ZO-1
Occludin
Claudin-1
Claudin-2
Claudin-4
Claudin-7
β-actin
220 kDa
65 kDa
25 kDa
25 kDa
25 kDa
25 kDa
42 kDa
B
ZO-1
Occludin
Claudin-1
Claudin-2
Claudin-4
Claudin-7
ZO-1/β-actin ratio
Occludin/β-actin ratio
Claudin-1/β-actin ratio
Claudin-2/β-actin ratio
Claudin-4/β-actin ratio
Claudin-7/β-actin ratio
C
ZO-1
Occludin
Claudin-1
Claudin-2
Claudin-4
Claudin-7
mRNA fold change

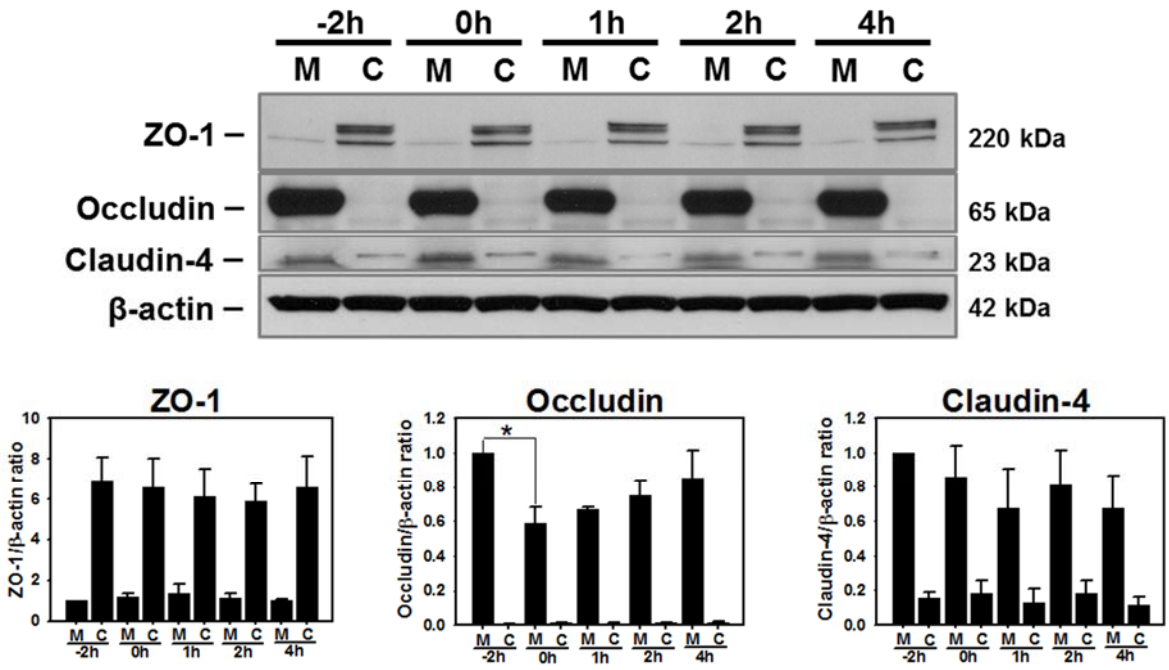
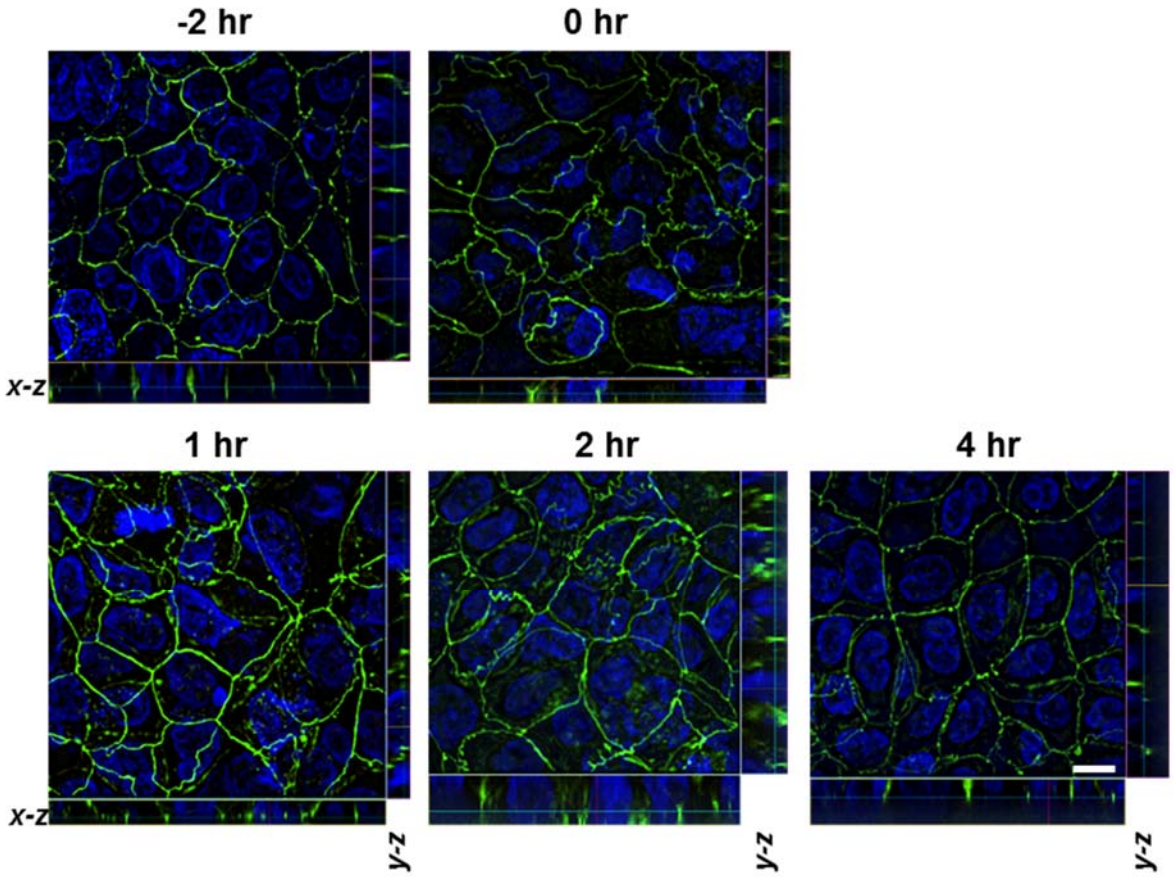


Figure 3. Control or hypoxic injured Caco-2BBe1 cells were analyzed by membrane and cytosol fractionated analysis for tight junction proteins ZO-1, occludin, and claudin-4. Expression of tight junction proteins were detected by immunoblotting. The expression of occludin in membrane fraction was significantly decreased after hypoxic injury and then recovered during the reoxygenation. Data are mean ± SEM (n=4 at each time point). *p<0.05, versus -2h.

Figure 4. Control and hypoxic injured Caco-2BBe1 cells were analyzed by immunofluorescence for tight junction protein occludin. Permeable support membranes were fixed and stained for occludin (green) and nuclei (blue) at indicated time point. Immunolocalization of occludin was analyzed by Z stack 3D analysis. Localization of sealing tight junction protein occludin was internalized after hypoxic injury. Bars =10 μm.


-2 hr
0 hr
x-z
1 hr
2 hr
4 hr
x-z
y-z
y-z
y-z

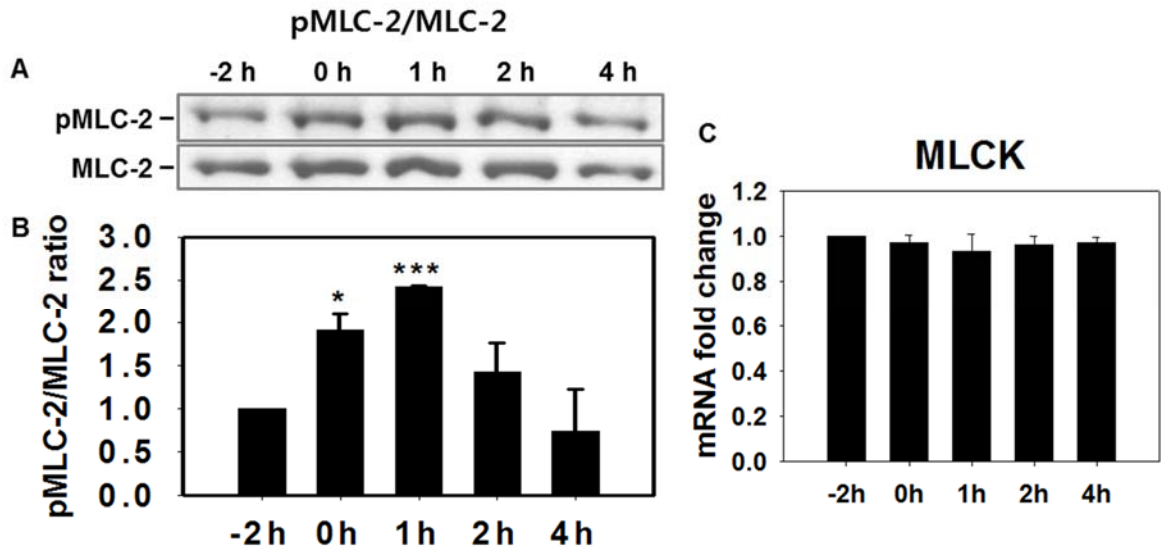
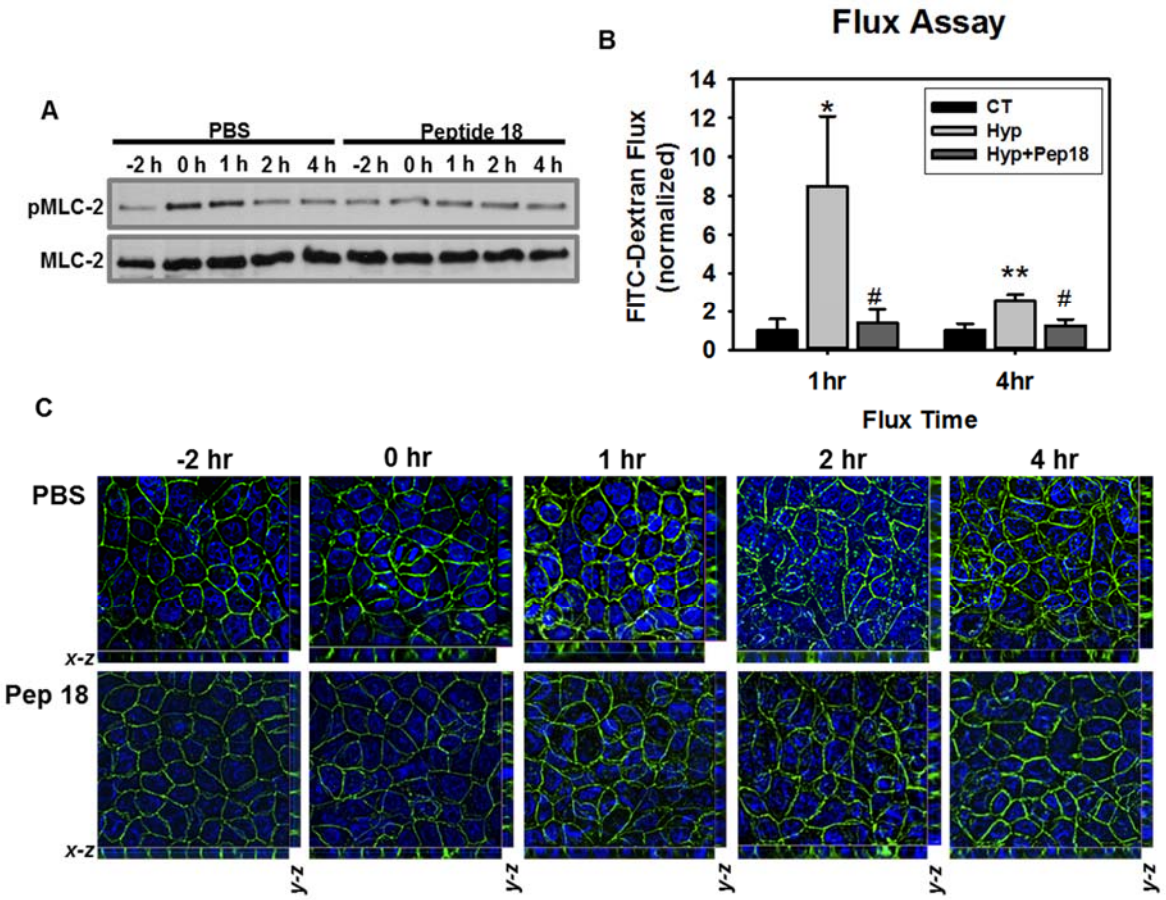


Figure 5. Hypoxic injury induced increased presence of pMCL-2 without change of MLCK expression. (A) Total cell lysate were analyzed for pMLC-2 and total MLC-2 by western blotting. The expression of pMLC-2 was increased after hypoxic injury. (B) Semi-quantitative analysis was performed with Image-Pro Plus software. Relative expression of pMLC-2 compared with total MLC-2 was significantly increased after hypoxic injury. (C) Real time PCR analysis of total cell lysate during H/R injury. The mRNA level of MLCK was not changed during H/R injury. Data are mean ± SEM (n=3-4 at each time point). *p<0.05, versus -2h.

Figure. 6 Effects of MLCK inhibition on TJ barrier function in H/R injury on the human intestinal cell monolayer. A. pMLC-2 and total MLC-2 were detected in total cell lysate by western blotting. Caco-2BBe1 cells treated with MLCK inhibitor peptide 18 (Pep 18) showed reduction in the expression of pMLC-2 during H/R injury compared with the untreated monolayer. B. Permeable support membranes treated with MLCK inhibitor Pep 18 resulted in decreased permeability of 4 kDa FITC-dextran compared with untreated monolayer. Data are mean ± SEM (n=5 at each time point). $*p<0.05$ $**p<0.01$, versus control. # $p<0.05$ versus hypoxia. C. Occludin endocytosis was reduced during the H/R injury after inhibition of MLCK.


A
PBS
Peptide 18
-2 h 0 h 1 h 2 h 4 h -2 h 0 h 1 h 2 h 4 h
pMLC-2
MLC-2
B
Flux Assay
FITC-Dextran Flux (normalized)
CT
Hyp
Hyp+Pep18
1hr
4hr
Flux Time
C
-2 hr
0 hr
1 hr
2 hr
4 hr
PBS
Pep 18
x-z
y-z

REFERENCES

1. Cerqueira NF, Hussni CA, Yoshida WB. Pathophysiology of mesenteric ischemia/reperfusion: a review. Acta cirurgica brasileira / Sociedade Brasileira para Desenvolvimento Pesquisa em Cirurgia 2005;**20**(4):336-43 doi: /S0102-86502005000400013[published Online First: Epub Date]|.
2. Pierro A, Eaton S. Intestinal ischemia reperfusion injury and multisystem organ failure. Seminars in pediatric surgery 2004;**13**(1):11-7
3. Turner JR. Intestinal mucosal barrier function in health and disease. Nature reviews. Immunology 2009;**9**(11):799-809 doi: 10.1038/nri2653[published Online First: Epub Date]|.
4. Shin K, Fogg VC, Margolis B. Tight junctions and cell polarity. Annual review of cell and developmental biology 2006;**22**:207-35 doi: 10.1146/annurev.cellbio.22.010305.104219[published Online First: Epub Date]|.
5. Ebnet K. Organization of multiprotein complexes at cell-cell junctions. Histochemistry and cell biology 2008;**130**(1):1-20 doi: 10.1007/s00418-008-0418-7[published Online First: Epub Date]|.
6. Kojima T, Murata M, Yamamoto T, et al. Tight junction proteins and signal transduction pathways in hepatocytes. Histology and histopathology 2009;**24**(11):1463-72
7. Shen L, Weber CR, Raleigh DR, et al. Tight junction pore and leak pathways: a dynamic duo. Annual review of physiology 2011;**73**:283-309 doi: 10.1146/annurev-physiol-012110-142150[published Online First: Epub Date]|.

8. Hassoun HT, Kone BC, Mercer DW, et al. Post-injury multiple organ failure: the role of the gut. Shock 2001;**15**(1):1-10

9. Kompan L, Kompan D. Importance of increased intestinal permeability after multiple injuries. The European journal of surgery = Acta chirurgica 2001;**167**(8):570-4 doi: 10.1080/11024150175317155[published Online First: Epub Date]|.

10. Li Q, Zhang Q, Zhang M, et al. Effect of n-3 polyunsaturated fatty acids on membrane microdomain localization of tight junction proteins in experimental colitis. The FEBS journal 2008;**275**(3):411-20 doi: 10.1111/j.1742-4658.2007.06210.x[published Online First: Epub Date]|.

11. Schmitz H, Barmeyer C, Fromm M, et al. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. Gastroenterology 1999;**116**(2):301-9

12. Marin ML, Geller SA, Greenstein AJ, et al. Ultrastructural pathology of Crohn's disease: correlated transmission electron microscopy, scanning electron microscopy, and freeze fracture studies. The American journal of gastroenterology 1983;**78**(6):355-64

13. Li Q, Zhang Q, Wang C, et al. Altered distribution of tight junction proteins after intestinal ischaemia/reperfusion injury in rats. Journal of cellular and molecular medicine 2009;**13**(9B):4061-76 doi: 10.1111/j.1582-4934.2009.00975.x[published Online First: Epub Date]|.

14. Moeser AJ, Nighot PK, Engelke KJ, et al. Recovery of mucosal barrier function in ischemic porcine ileum and colon is stimulated by a novel agonist of the ClC-2 chloride channel, lubiprostone. American journal of physiology. Gastrointestinal and

liver physiology 2007;**292**(2):G647-56 doi: 10.1152/ajpgi.00183.2006[published Online First: Epub Date]|.

15. Nighot PK, Moeser AJ, Ryan KA, et al. ClC-2 is required for rapid restoration of epithelial tight junctions in ischemic-injured murine jejunum. Experimental cell research 2009;**315**(1):110-8 doi: 10.1016/j.yexcr.2008.10.001[published Online First: Epub Date]|.
16. Moeser AJ, Haskell MM, Shifflett DE, et al. ClC-2 chloride secretion mediates prostaglandin-induced recovery of barrier function in ischemia-injured porcine ileum. Gastroenterology 2004;**127**(3):802-15
17. Nighot PK, Blikslager AT. Chloride channel ClC-2 modulates tight junction barrier function via intracellular trafficking of occludin. American journal of physiology. Cell physiology 2012;**302**(1):C178-87 doi: 10.1152/ajpcell.00072.2011[published Online First: Epub Date]|.
18. Blikslager AT, Moeser AJ, Gookin JL, et al. Restoration of barrier function in injured intestinal mucosa. Physiological reviews 2007;**87**(2):545-64 doi: 10.1152/physrev.00012.2006[published Online First: Epub Date]|.
19. Little D, Dean RA, Young KM, et al. PI3K signaling is required for prostaglandin-induced mucosal recovery in ischemia-injured porcine ileum. American journal of physiology. Gastrointestinal and liver physiology 2003;**284**(1):G46-56 doi: 10.1152/ajpgi.00121.2002[published Online First: Epub Date]|.
20. Shen L, Black ED, Witkowski ED, et al. Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure. Journal of cell science

2006;**119**(Pt 10):2095-106 doi: 10.1242/jcs.02915[published Online First: Epub Date]|.

21. Clayburgh DR, Barrett TA, Tang Y, et al. Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo. The Journal of clinical investigation 2005;**115**(10):2702-15 doi: 10.1172/JCI24970[published Online First: Epub Date]|.
22. Yuan SY, Rigor RR. Regulation of Endothelial Barrier Function. San Rafael (CA), 2010.
23. Su L, Nalle SC, Shen L, et al. TNFR2 activates MLCK-dependent tight junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis. Gastroenterology 2013;**145**(2):407-15 doi: 10.1053/j.gastro.2013.04.011[published Online First: Epub Date]|.
24. Schwarz BT, Wang F, Shen L, et al. LIGHT signals directly to intestinal epithelia to cause barrier dysfunction via cytoskeletal and endocytic mechanisms. Gastroenterology 2007;**132**(7):2383-94 doi: 10.1053/j.gastro.2007.02.052[published Online First: Epub Date]|.
25. Camilleri M, Madsen K, Spiller R, et al. Intestinal barrier function in health and gastrointestinal disease. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 2012;**24**(6):503-12 doi: 10.1111/j.1365-2982.2012.01921.x[published Online First: Epub Date]|.
26. Van Itallie CM, Fanning AS, Holmes J, et al. Occludin is required for cytokine-induced regulation of tight junction barriers. Journal of cell science 2010;**123**(Pt 16):2844-52 doi: 10.1242/jcs.065581[published Online First: Epub Date]|.

27. Marchiando AM, Shen L, Graham WV, et al. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. The Journal of cell biology 2010;**189**(1):111-26 doi: 10.1083/jcb.200902153[published Online First: Epub Date]|.

28. Wang F, Graham WV, Wang Y, et al. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. The American journal of pathology 2005;**166**(2):409-19

29. Ma TY, Boivin MA, Ye D, et al. Mechanism of TNF-{alpha} modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. American journal of physiology. Gastrointestinal and liver physiology 2005;**288**(3):G422-30 doi: 10.1152/ajpgi.00412.2004[published Online First: Epub Date]|.

30. Hu Q, Ziegelstein RC. Hypoxia/reoxygenation stimulates intracellular calcium oscillations in human aortic endothelial cells. Circulation 2000;**102**(20):2541-7

31. Furuta GT, Turner JR, Taylor CT, et al. Hypoxia-inducible factor 1-dependent induction of intestinal trefoil factor protects barrier function during hypoxia. The Journal of experimental medicine 2001;**193**(9):1027-34

32. Colgan SP, Taylor CT. Hypoxia: an alarm signal during intestinal inflammation. Nature reviews. Gastroenterology & hepatology 2010;**7**(5):281-7 doi: 10.1038/nrgastro.2010.39[published Online First: Epub Date]|.

33. Taylor CT, Colgan SP. Hypoxia and gastrointestinal disease. Journal of molecular medicine 2007;**85**(12):1295-300 doi: 10.1007/s00109-007-0277-z[published Online First: Epub Date]|.