2012

The Effects of Enteropathogenic and Commensal Escherichia Coli on Tight Junction Permeability

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THE EFFECTS OF ENTEROPATHOGENIC AND COMMENSAL *ESCHERICHIA COLI* ON TIGHT JUNCTION PERMEABILITY

A thesis submitted in partial fulfillment of the

requirements for the degree of

Master of Science

By

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B.S., Wright State University, 2009

2012
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

May 30, 2012

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY _Hilary Kaye Allen_ ENTITLED The Effects of Enteropathogenic and Commensal *Escherichia coli* on Tight Junction Permeability BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Allen, Hilary Kaye. M.S., Microbiology and Immunology, Wright State University, 2012. The Effects of Enteropathogenic and Commensal *Escherichia coli* on Tight Junction Permeability.

The intestinal mucosa maintains a barrier between materials from the external environment and the internal environment of the host. Disruption of the gut wall integrity is involved in the development of various intestinal diseases, such as Irritable Bowel Syndrome (IBS) and Crohn's disease. The intestinal mucosa is lined with epithelial cells that are connected by tight junctions, the intercellular junctions that form a selectively permeable barrier between paracellular pathways. Enteric pathogens, such as enteropathogenic *Escherichia coli* (EPEC), can disrupt the tight junctions of epithelial cells by altering the cellular cytoskeleton or by directly affecting tight junction proteins. Commensal *Escherichia coli* can also modify intestinal epithelial barrier function, however, the role of commensal *E. coli* in tight junction permeability is not fully understood. Here, the effects of enteropathogenic and commensal *E. coli* on intestinal epithelial barrier integrity, with a focus on tight junction permeability, will be discussed.

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I. INTRODUCTION

The intestinal mucosa maintains a barrier between materials from the external environment and the internal environment of the host (1). Disruption of the gut wall integrity is involved in the development of various intestinal diseases, such as Irritable Bowel Syndrome (IBS) and Crohn's disease and plays a major role in the onset of sepsis and multiple organ failure (2). The defense mechanisms of the intestinal epithelial barrier consist of a physical and immunological component (2). The physical barrier is lined with epithelial cells that are connected by tight junctions, which will be the main focus of discussion (2).

Tight junctions are adhesion structures that form a selectively permeable barrier between paracellular pathways (2). Enteric pathogens disrupt epithelial barrier function through alteration of tight junctions via various mechanisms, for example, through modification of the cellular cytoskeleton or redistribution of tight junction proteins (3). The disruption of tight junctions by enteric pathogens is generally measured by a reduction in transepithelial resistance (TER) or an increase in paracellular flux of macromolecules (3).

Many enteropathogenic bacteria have been implicated in the disruption of tight junctions including enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Clositridium difficile, Clostridium perfringens, Helicobacter pylori, Campylobacter jejuni, Campylobacter concisus,* and *Salmonella typhimurium* (3). Several of these bacteria disrupt tight junctions through disorganization of specific tight junction proteins, including zonula occludens, occludin, and claudin (4). The mechanisms by which EPEC

affect tight junction barrier function are discussed here.

Enteropathogenic *Escherichia coli* (EPEC) are gram-negative bacteria that cause diarrhea and significant infant mortality in developing countries (5). EPEC are characterized by the attaching and effacing (A/E) lesions observed during infection (Figure 1). EPEC intimately attach to pedestal-like projections of the apical enterocyte membrane, followed by destruction of microvilli (Figure 1). The A/E lesions are believed to adhere EPEC to the cell surface, as EPEC remains extracellular during pathogenesis (5).

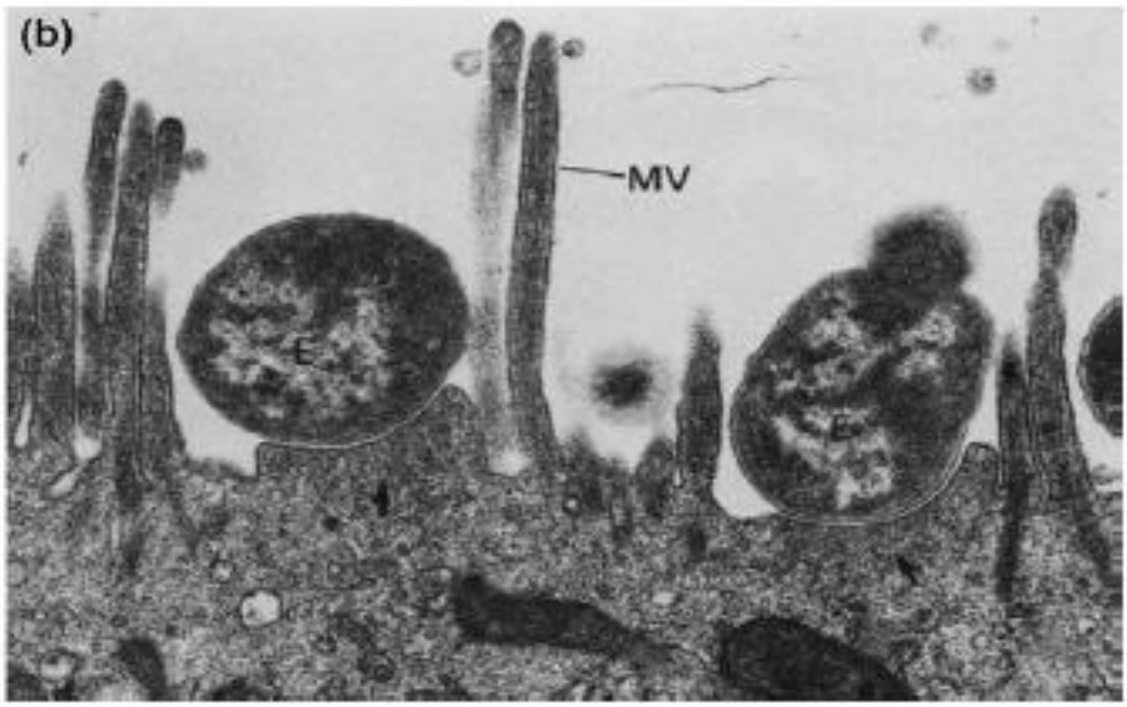

Figure 1. Electron micrograph of EPEC infected human intestinal mucosa. Adapted from Chen et al. 2005

EPEC secretes various proteins during infection via the type three secretion system (TTSS) (Figure 2). The TTSS is utilized by a variety of enteric pathogens and

acts as a syringe injecting effector proteins into host cells (Figure 3). In contrast to commensal *E. coli*, EPEC contains a 35.6 kb pathogenicity island known as the locus of enterocyte effacement (LEE) that is also involved in EPEC virulence (5).

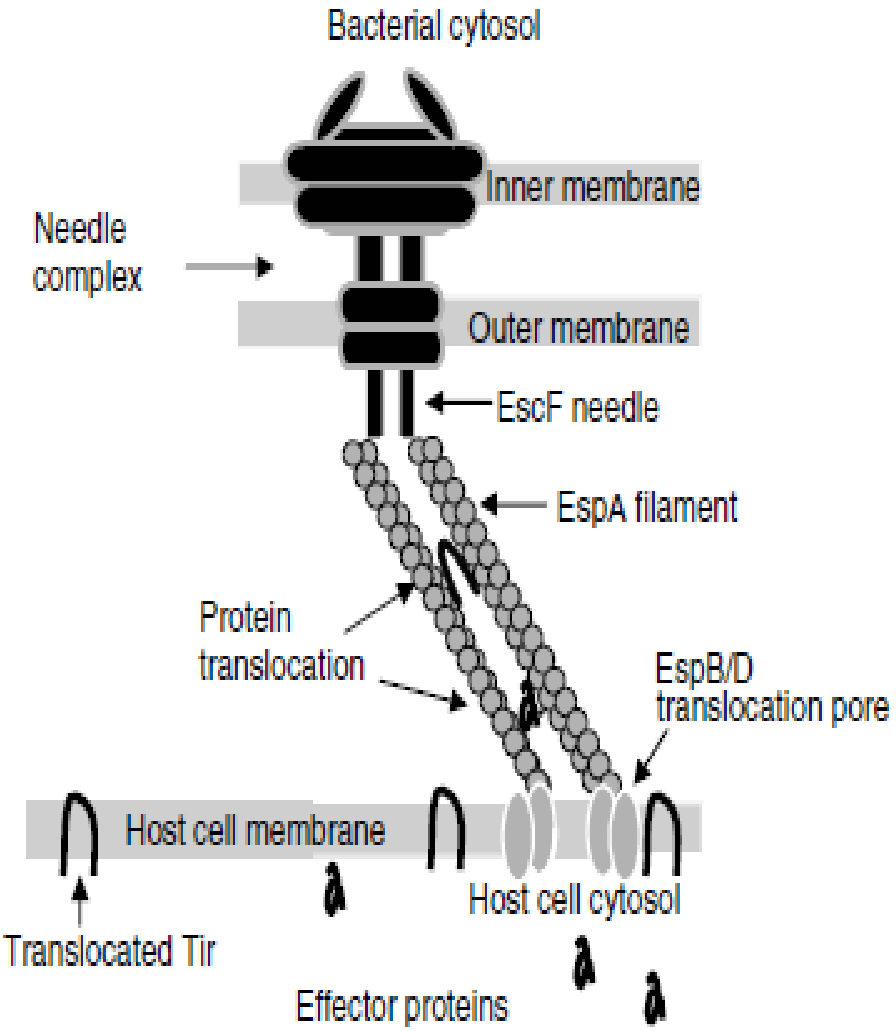

Figure 2. Type III protein translocation in EPEC. Adapted from Chen et al. 2005

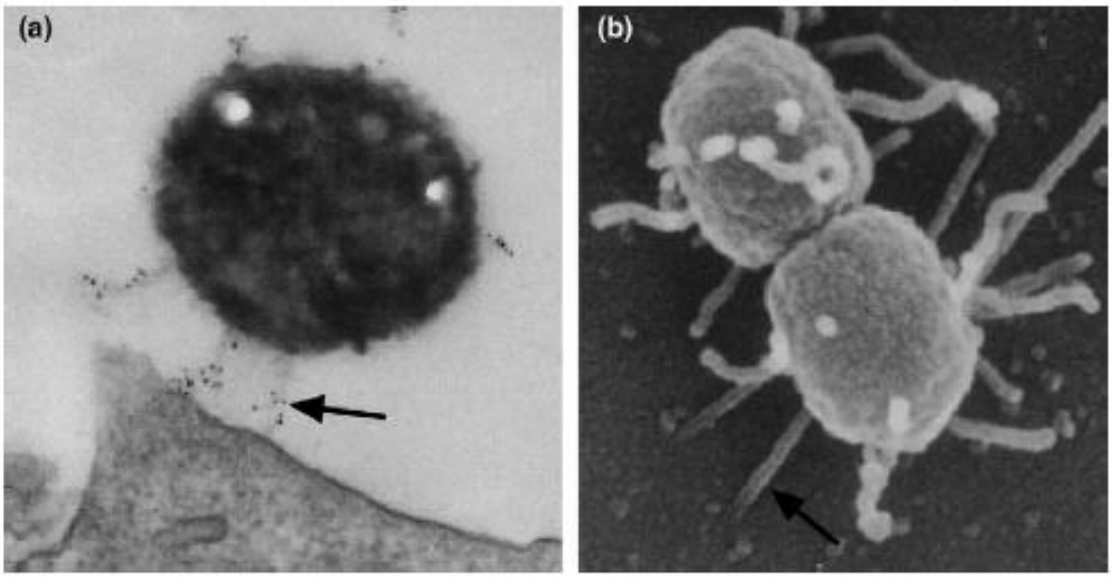

Figure 3. A. Effector protein filaments (black arrows) forming a bridge between bacteria and epithelial cells during early stages of A/E lesion formation. B. Effector protein filaments (black arrows) connecting EPEC bacteria to the plasma membrane enabling injection of translocated effector proteins. Adapted from Chen et al. 2005.

The adult human gut is home to approximately 10^{14} bacterial cells, with each host possessing a unique composition of bacterial species (6). The gastrointestinal tract is covered by mucus that is secreted by goblet cells (7). The small intestine has only one layer of mucus, compared to the two layers in the stomach and colon (7). The production of mucins provides an important energy source for commensal bacteria within the gut (7).

The commensal intestinal microbiota plays a key role in promoting a healthy environment within the host, including a healthy intestinal pH, immune homeostasis, and metabolism (6). Commensal bacteria can also prevent the colonization of pathogenic bacteria by competing for space (8).

An important part of the gut immunity is distinguishing between commensal and

pathogenic bacteria (9). This is extremely important in the intestine, where the immune system is continually challenged with microorganisms without inducing an inflammatory response (9). A key component of distinguishing non-pathogenic vs. pathogenic organisms in the host is intestinal mononuclear phagocytes (iMPs) (9). The iMPs that generally reside in the intestinal lamina propria (macrophages and dendritic cells) are hyporesponsive to bacterial stimulation, which may account for a lack of inflammatory response to normal gut microbiota (9).

Commensal *Escherichia coli*, a rod-shaped, gram-negative facultative anaerobe, are an important component of the gut microflora (6). Changes in concentration of commensal *E. coli* have been implicated in various intestinal diseases such as Crohn's disease (6). For example, Packey et al. described several ways in which commensal enteric bacteria induce and maintain chronic inflammation in the intestines associated with inflammatory bowel disease (IBD): abnormal microbial composition, defective host containment of commensal bacteria, and defective host immunoregulation (6).

Modulations of the gut microbiota have also been shown to increase or decrease epithelial barrier integrity by altering the expression of tight junction proteins (8). For example, modulating the gut microbiota with a dietary supplement of a prebiotic resulted in increased expression of the tight junction proteins ZO-1 and occludin, thereby increasing intestinal epithelial barrier integrity (8).

Both enteropathogenic *E. coli* and commensal *E. coli* have been shown to modify tight junction permeability. EPEC-induced disruption of tight junctions has been studied extensively *in vitro* and *in vivo*, with a general focus on alteration of tight junction

proteins (10). For example, Muza-Moons et al. demonstrated the interactions between the tight junction proteins occludin and claudin with ZO-1 are lost in response to EPEC infection (10). In contrast, a great deal less is known about the role of commensal *E. coli* in tight junction permeability. Here, the effects of enteropathogenic and commensal *E. coli* on intestinal epithelial barrier integrity will be discussed, with a focus on tight junction permeability.

II. BACKGROUND

1. Intestinal Epithelial Cells

Polarized epithelial cells are located between the lumen and the deep cell layers of the organs (Figure 4). The epithelial cells and the lamina propria (a constituent of membrane linings composed of a thin layer of connective tissue) make up the intestinal mucosa (1). Epithelial stem cells develop into four major types of epithelial cells; enterocytes, goblet cells, enteroendocrine cells, and Paneth cells (1). The enterocytes of the intestinal epithelium will be the main focus of discussion here.

Intestinal epithelial cells display distinct apices and bases, where they anchor into the extracellular matrix (11). Neighboring cells attach to one another laterally through intercellular junctions (11). The apical junction complex, consisting of the tight junction, adherens junction, and desmosome, is located at the most apical lateral plasma membrane (11).

The tight junctions and adherens junctions attach to actin filaments through cytoplasmic adaptor proteins and are involved in many signaling pathways (11). The

components of the apical junction complex contain various transmembrane proteins, cytoskeletal elements, and cytoplasmic scaffolding proteins, all of which work together to prevent luminal material from entering tissues (11).

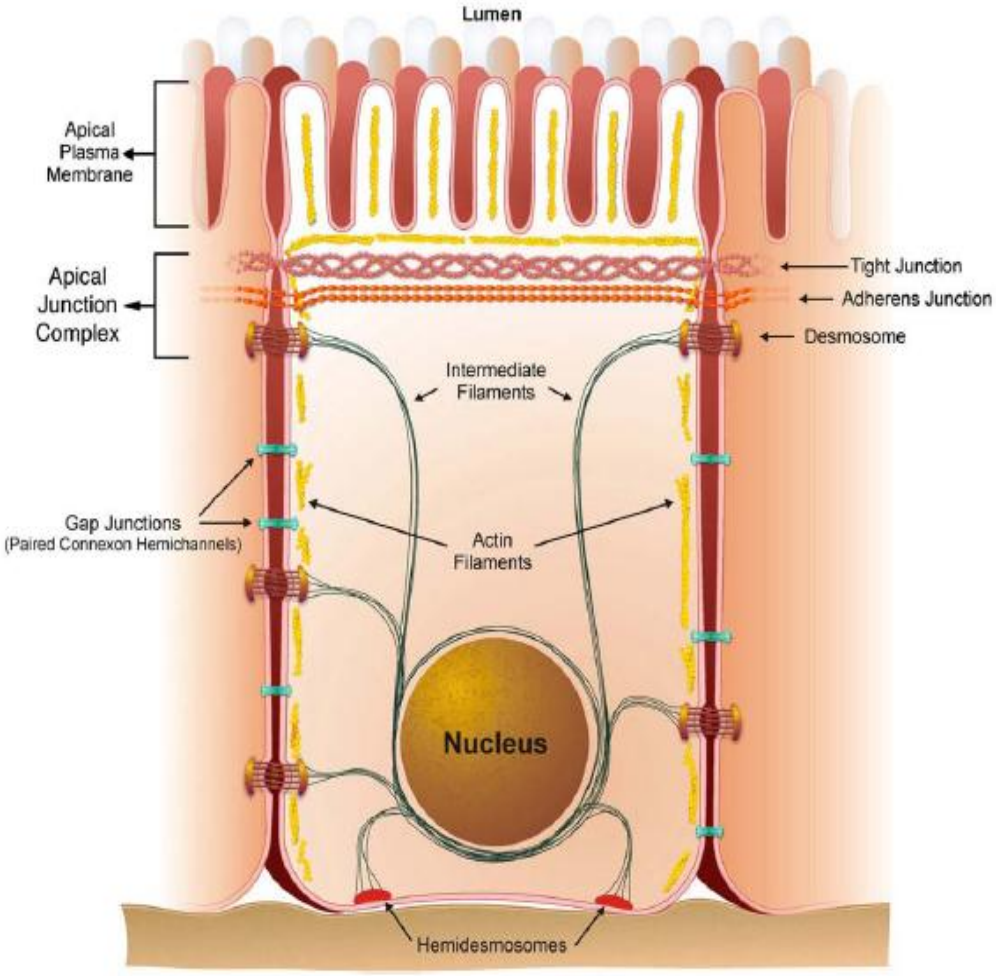

Figure 4. Representation of junctions in polarized epithelial cells. Adapted from Guttman et al. 2009.

2. Tight Junction Structure and Function

Tight junctions are the most apically located of the intercellular junctions that

function as the primary barrier to the diffusion of solutes through the paracellular pathway (12). Tight junctions are found in epithelial cell types, including endothelial cells, mesothelial cells, and other types of cells such as Schwann cells, oligodendrocytes, and Sertoli cells (12, 13). Observation of tight junctions by transmission electron microscopy reveals a series of membrane fusions between adjacent cells, often referred to as "kisses" (13).

When viewing tight junctions with freeze-fracture electron microscopy, the "kisses" appear as networks of intramembranous particle fibrils, or tight junction strands (Figure 5). As tight junctions are a semipermeable barrier to ion, solute, and water transport, they are involved in the coordination of many signaling and trafficking molecules (13). The signaling pathways include regulation of cellular differentiation, proliferation, and polarity, all of which are crucial in establishing tissue compartments and homeostasis within the body (13).

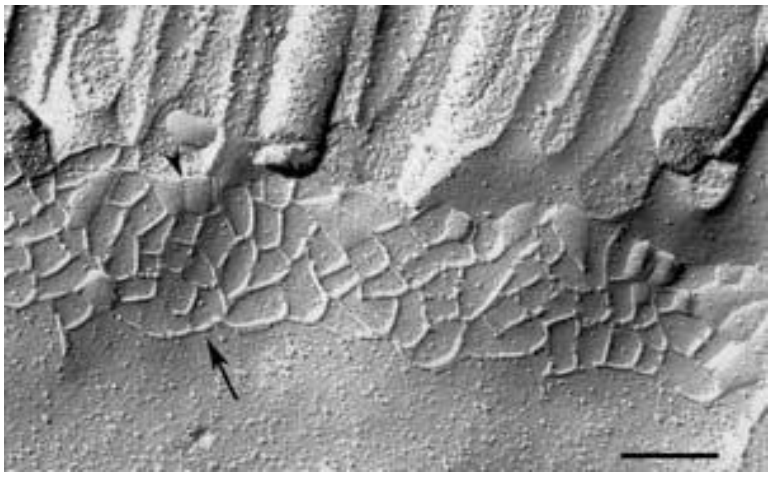

Figure 5. Freeze fracture image of tight junctions in mouse intestinal epithelial cells. The tight junctions appear as fibrils, or tight junction strands, as indicated by the arrow. Adapted from Furuse et al. 2012.

As expected, disruption of tight junctions is implicated in many intestinal diseases, such as inflammatory bowel disease (IBD) (13). Furthermore, previous studies have demonstrated the various transmembrane proteins of tight junctions are altered and translocated during intestinal diseases and bacterial infections (13). For example, the interferon-gamma (IFN-γ) induced internalization of the tight junction proteins claudin-1 and -4 in T84 cells may contribute to barrier dysfunction in IBD (13).

3. Tight Junction Proteins

The first identified protein of tight junctions, occludin, is a 60-kDa protein containing two extracellular loops (See figure 6 for an overview of tight junction proteins) (13). The C-terminal domain of the long loop of occludin is abundant in serine, threonine, and tyrosine residues that are phosphorylated by different protein kinases (13). The C-terminal region also binds directly to ZO-1, a junctional adhesion molecule that associates with the actin cytoskeleton (Figure 6).

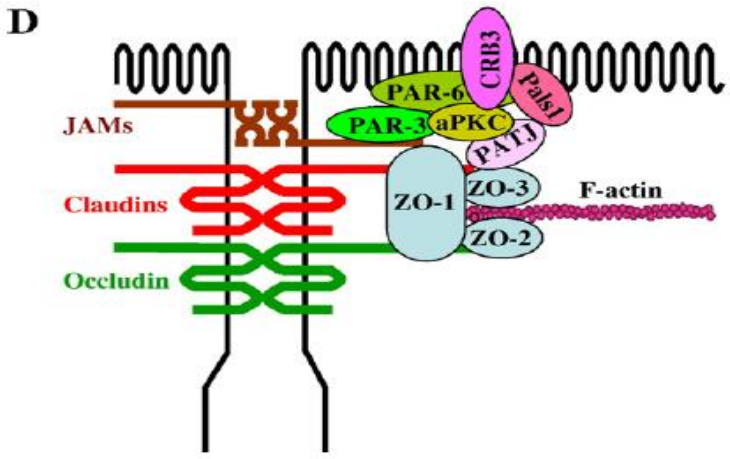

Figure 6. The molecular components of tight junctions. Adapted from Chiba et al., 2008.

Occludin was originally believed to play a role in barrier function; however, a previous study showed occludin-deficient embryonic stem cells developed into epithelial cells with functioning tight junctions (13). Other studies have shown occludin may play a part in tight junction formation and cell adhesion, for example, in MDCK cells occludin induced an increase in TER (14). As the function of occludin is still not yet fully understood, extensive analysis of the protein will be necessary in future studies.

The next important member of the tight junction family is claudin. Claudins are generally 18- to -27kDa proteins and contain a short N-terminus, two extracellular loops, and a C-terminal domain (13). The claudin family consists of 24 members, and is considered the "backbone" of the tight junction (13). Claudins can also directly bind to PDZ domain-possessing proteins such as ZO-1, ZO-2, and ZO-3 (Figure 7).

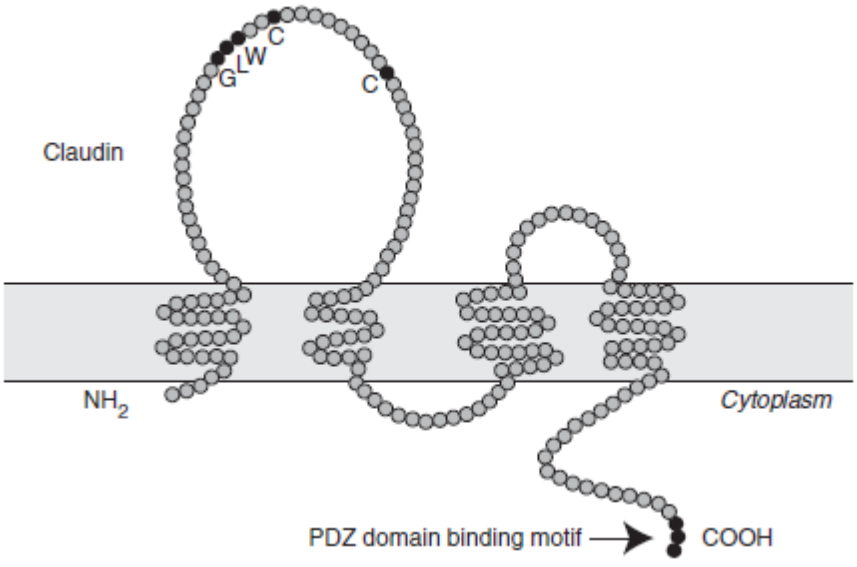

Figure 7. A membrane-spanning model of the Claudin protein. The PDZ domain binding motif is located at the carboxyl terminus. Adapted from Future et al. 2010.

Claudin proteins are expressed in most types of epithelial cells and are considered a major determinant of tight junction barrier functions, such as paracellular charge and size selectivity (13). In claudin proteins, unlike occludin, paracellular pores (channels) are created for ions between adjacent cells, a process mediated within the first extracellular loop (13).

Altered claudin expression and distribution has been implicated in a number of human diseases. For example, the internalization of claudin-1 and -4 is believed to be involved in the barrier dysfunction in IBD in the human intestinal epithelial T84 cells (13). Although, a limitation within the study was cell line specificity, as IBD is a systemic disease. Studies have also shown an increased expression of claudin proteins in various types of cancers (13).

The last member of the tight junction family discussed here is the zonula occludens (ZO). Within tight junctions, PDZ domain-containing cytoplasmic proteins interact with integral membrane proteins to form cytoplasmic plaques (12). The plaques work as scaffolds to recruit other proteins and the actin cytoskeleton to the surface of tight junctions (12). The proteins ZO-1, ZO-2, and ZO-3 are part of these cytoplasmic plaques (12).

As mentioned before, the domains of the ZO proteins directly bind to the C-terminus of claudins (12). ZO-1 is also capable of binding to occludin and junctional adhesion molecule-A (JAM-A), an additional tight junction-associated integral membrane protein) (12). The ZO proteins can also interact with actin filaments (12).

ZO-1 and ZO-2 are believed to be absolutely necessary for tight junction

formation in epithelial cells (12). For example, disruption of the ZO-1 gene and depletion of the ZO-2 protein led to deficient tight junction formation in mouse epithelial cells (12). ZO-1 is also believed to induce claudin polymerization into tight junction strands (12).

III. THE EFFECTS OF COMMENSAL *ESCHERICHIA COLI* ON TIGHT JUNCTION PERMEABILITY

1. Commensal Bacteria as a Threat to the Epithelium Under Metabolic Stress

Metabolic stress may play a pivotal role in the pathophysiology of gut diseases such as inflammatory bowel disease (IBD) (15). Currently, there is no cure for IBD, and most therapies are not ideal given the possible side effects (13). It is hypothesized that IBD is caused by an inappropriate immune response to the normal flora of the gut, and different stressors exacerbate these effects on epithelial permeability (15).

Nazli et al. used the chemical stressor dinitrophenol (DNP, an uncoupler of oxidative phosphorylation) to investigate the effects of commensal *E. coli* on epithelial permeability during metabolic stress in rats (15). The authors hypothesized that when enteric epithelia are under metabolic stress, they will "perceive" normal gut bacteria as a threat, leading to a loss of barrier function, increased translocation of bacteria into the mucosa, and increased chemokine synthesis (15).

The experimental methods consisted of injecting DNP into the ileal lumen of rats (15). Segments of the ileum were removed 6 or 24 hours later and portions of the tissue were fixed and stained with hematoxylin and eosin (H&E) (15). Portions of the tissue

were then mounted in Ussing chambers (an instrument that measures short-circuit current as an indicator of net ion transport across an epithelium) in order to measure barrier function (15).

The results of the study showed DNP treatment induced an increase in epithelial permeability, as measured by ion conductance and transepithelial flux (15). There was also increased immune cell traffic to the gut in the 6 to 24-hour period, as indicated by an increased amount of immune cells, including mononuclear cells and granulocytes, in the mucosa (15). There were approximately 130 mononuclear cells/mm² with DNP at 6 hours after treatment compared to approximately 70 cells/mm² in untreated controls (15)

The cell lines used for the *in vitro* cell culture studies were human colon-derived crypt-like T84 and HT-29 epithelial cell lines (15). Non-pathogenic *E. coli* strains HB101 and C25 were cultured and added to, along with DNP, filter-grown monolayers (15). The control systems consisted of time-matched naïve monolayers, DNP only, and *E. coli* HB101 or C25 only (15). Enteropathogenic *E. coli* was used as a positive control for bacterial disruption of epithelial barrier function (15).

After cell monolayers were exposed to DNP and nonpathogenic *E. coli* HB101, a significant increase in epithelial permeability (represented as a decrease in TER) and an increase in transepithelial flux occurred (15). In the control group, TER (presented as a percent of pretreatment values) was approximately 100% and in the DNP+HB101 treated monolayers TER was approximately 60% after 24 hours of exposure (13). Exposure of the monolayers to DNP and nonpathogenic *E. coli* alone did not affect TER (15).

The results were slightly different when *E. coli* strain C25 was administered, as the strain induced a decrease in TER without the addition of DNP (13). When combined with DNP, TER decreased even further (15). The results indicate *E. coli* C25 as a possible low-grade-pathogen and stressed epithelia are more sensitive to this strain of *E. coli* (15).

Nazli et al. also demonstrated that the increase in epithelial permeability induced by DNP + *E. coli* HB101 was due to the impact of DNP on the epithelium and not a result of a bacteria-derived product (15). The combination of DNP + *E. coli* HB101 also resulted in significant bacterial translocation across filter-grown epithelial monolayers (15). The increase in bacterial translocation was not observed when monolayers were treated with *E. coli* HB101 only (15).

Together, the experiments by Nazli et al. demonstrated a strain of commensal *E. coli*, when coupled with a chemical stressor, can disrupt epithelial barrier function *in vitro* (15). Previous studies have shown altered energy metabolism in gut tissues from patients with IBD, for example, decreased ATP levels were found in inflamed tissue excised from IBD patients (15). Epithelial barrier maintenance is dependent on regulation of the tight junctions, which is an energy-dependent process (15). Therefore, it is of interest to investigate alterations in epithelial barrier function during metabolic stress in association with the normal gut flora.

Nazli et al. demonstrated a link between commensal bacteria and an alteration in epithelial barrier function during metabolic stress (15). The epithelium may perceive the normal gut flora as a threat during stress (15). This link may be implicated during certain

inflammatory bowel diseases such as Crohn's disease, and ulcerative colitis (15). Future studies should include a more detailed investigation into what proteins of the tight junctions are altered, by measuring the expression levels of occludin, claudin, or ZO-1.

Nazli et al. analyzed the specific tight junction proteins that were altered as a result of a DNP+*E. coli* HB101 induced decrease in paracellular permeability (16). The study included a more structural assessment of the effects of nonpathogenic *E. coli* and DNP on tight junction protein expression, transepithelial resistance (TER), and bacterial translocation (16). Nazli et al. demonstrated enteric epithelial cells experiencing stress, or an altered energy balance, are capable of endocytosing commensal bacteria that can subsequently cross the epithelial layer (16).

The experimental methods involved treating confluent filter-grown monolayers of the human colonic T84 epithelial cell line with 0.1 mM DNP and nonpathogenic *E. coli* HB101 with or without pretreatment with pharmacological agents (16). Transepithelial resistance was measured after 5 and 7 days of culture with a voltmeter and matched electrodes (16). Flux assays were performed by adding horseradish peroxidase (HRP) to the apical side of the filter-grown T84 monolayers (16). Intact HRP was determined by kinetic enzymatic assay as the amount of HRP recovered compared to the initial concentration (16).

For the analysis of tight junction proteins, protein concentrations were measured using a microplate assay. Next, SDS loading buffer was added to each sample and separated proteins were electroblotted (16). The blots were washed and incubated with

the following tight junction proteins: ZO-1, occludin, claudin-1, claudin-2, and claudin-4 (16).

The four treatment groups used in the study were as follows: naïve controls, cells receiving pharmacological agent treatment only, DNP + *E. coli* HB101 (the positive control), and DNP + *E. coli* HB101 + pharmacological agent (the test condition) (16). As previous studies have shown microtubule architecture to play a role in paracellular permeability, it was also important to investigate whether the DNP and *E. coli* HB101-induced decrease in TER was diminished by microtubule or microfilament stabilization (16). Nazli et al. demonstrated that cytochalasin D (a depolymerizing drug) did not prevent a drop in TER caused by a 24h culture with DNP + *E. coli* HB101 (16).

As a reduction in TER is associated with altered tight junction structure and more "open" tight junctions, Nazli et al. investigated the effects of DNP and *E. coli* HB101 on specific tight junction proteins (16). The results showed DNP and *E. coli* HB101 alone affected the expression of actin, occludin, and ZO-1 (a decrease in expression compared to the uninfected control), although the effects were not statistically significant (16).

However, in T84 cells treated previously with DNP + *E. coli* HB101, the expression of actin, occludin, and ZO-1 were all significantly reduced (16). There were no changes detected in the protein levels of claudin-1, claudin-2, and claudin-4 (16). This may be due to the fact that claudin proteins are believed to be more adhesive than occludin proteins, and therefore it is more difficult to alter expression levels during epithelial disruption (11, 12, and 16).

Next, Nazli et al. investigated whether blocking endocytosis would reduce bacterial internalization and translocation (16). Using methyl-β-cyclodextrin (MβCD) and phenylarside oxide (PAO), membrane cholesterol level and clathrin-coated pit formation reducing agents, respectively, there was no statistically significant abolishment of the DNP+*E.coli* HB101-induced drop in TER (16). However, cotreatment with MβCD or PAO inhibited bacterial internalization (16). MβCD reduced bacterial internalization by 85% compared to T84 cells treated with DNP+*E. coli* only, and PAO reduced bacterial internalization by approximately 45% (16).

Overall, the results of the study contribute to previous indications that nonpathogenic bacteria can increase gut paracellular permeability (16). Commensal bacteria may possess disease-promoting capabilities, especially during times of intestinal stress, such as reduced intestinal epithelial barrier integrity (16). A reduction in TER is indicative of altered tight junction structure, which is highlighted by the immunoblot analysis that showed a decrease in expression of various tight junction proteins in DNP+*E. coli* HB101 treated cells (16).

It is also important to note that DNP or *E. coli* HB101 alone caused a lesser degree of reduction in expression level of the same tight junction proteins (16). Therefore, in order for commensal bacteria to induce a decrease in epithelial barrier function, an additional perturbation of the enterocyte may be necessary (16).

Pharmacological agents used to block rearrangements of the enterocyte cytoskeleton or endocytosis also did not abrogate the DNP + *E. coli* HB101-induced drop

in TER (16). These observations may indicate a direct effect on tight junction proteins and not cytoskeletal interference (16). In contrast, interference with the cytoskeleton has been previously described during EPEC infection (16).

In conclusion, an alteration in epithelial energy balance (caused by DNP) may lead to the internalization of nonpathogenic bacteria in association with an increase in epithelial permeability (16). Next, it will be crucial to determine the signaling pathways involved in the alteration of the tight junction protein expression (16). Future studies of this nature will be important for providing evidence of commensal bacteria triggering and exacerbating chronic intestinal inflammatory diseases (16).

As commensal bacteria do not usually invade enterocytes, the underlying mechanisms by which commensal bacteria cross the epithelial barrier under conditions of stress and various inflammatory disorders is still not fully understood (17). Previous studies have implicated the release of proinflammatory cytokines, such as interferon gamma (IFN-γ) as contributors to gut barrier impairment (17).

Clark et al. investigated the mechanisms of IFN-γ mediated bacterial translocation across human colonic T84 monolayers (17). The proposed mechanism is commensal bacteria enter the body through "leaky" tight junctions, which can potentially lead to the development of disorders such as inflammatory bowel disease and sepsis (17). Clark et al. investigated the role of nonpathogenic *Escherichia coli* strain C25 in translocation across epithelial monolayers exposed to IFN-γ (17). Clark et al. showed IFN-γ-mediated translocation of *E. coli* C25 is a transcellular process involving lipid rafts, as opposed to an alteration in paracellular permeability and tight junction disruption (17).

The experimental methods included *E. coli* C25 translocation across Caco-2 and T84 cell colonic epithelial monolayers, which were exposed to varying concentrations of IFN-γ for 48 hours (17). The TER of the monolayers was measured at the end of the time period and tight junction protein expression was analyzed with immunoblotting and after band visualization, stained with Coomassie blue to confirm equal protein loading in each well (17). The tight junction proteins analyzed in the study were occludin, ZO-1, and claudin-1 (17).

The methods of immunofluorescence included cells cultured on Transwell supports and exposed to medium alone or IFN-γ and then visualized by confocal laser scanning microscopy (17). Addition of IFN-γ to the T84 colonic cells resulted in increased paracellular permeability, measured by a loss of TER and increased permeability to the paracellular probe LY (17). The permeability effects were dose dependent, with significant changes at 10 IU/mL IFN-γ (17).

In addition, treatment with IFN-γ resulted in a dramatic increase in translocation of *E. coli* C25 from the apical to the basolateral compartment (17). The effects of IFN-γ on *E.coli* C25 translocation across colonic epithelial monolayers were assessed with Transwell culture inserts that were exposed to varying concentrations of IFN-γ (0-100IU/mL) for 48 hours (17). The translocation of *E. coli* C25 occurred at a much lower IFN-γ concentration (1 IU/mL) than what was necessary for a permeability increase (17).

The immunoblot analysis of tight junction proteins demonstrated an uncoupling of IFN-γ-mediated changes in bacterial paracellular permeability and translocation (17). Occludin expression decreased in detergent-insoluble and soluble membrane fractions

from T84 cells after exposure to 100 IU/mL IFN-γ (Figure 4). The same concentration of IFN-γ lead to an increase in claudin-1 expression compared to untreated controls (Figure 8). However, at 1 IU/mL IFN-γ, the same concentration that *E. coli* C25 translocation was highly stimulated, both tight junction proteins occludin and claudin did not show any change in expression or distribution compared to the untreated control (Figure 8).

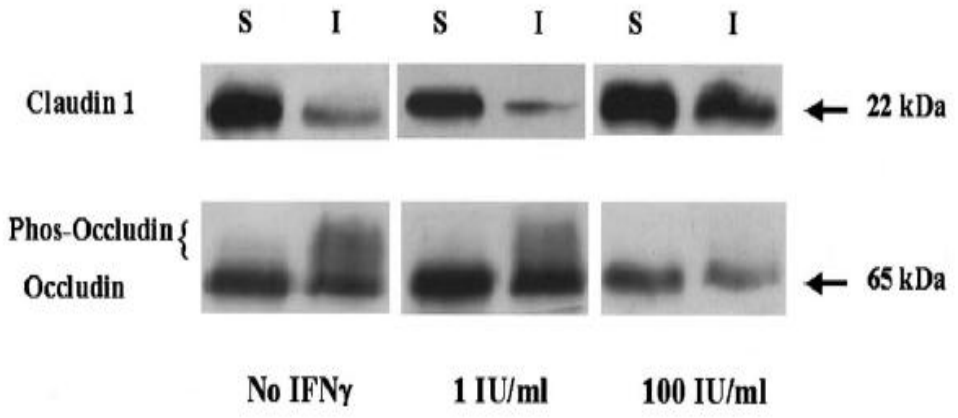

Figure 8. The effects of IFN-γ on tight junction proteins occludin and claudin-1 in T84 cells. Protein expression was determined in detergent-soluble (S) and detergent-insoluble (I) membrane fractions. Adapted from Clark et al. 2005

Similar results were obtained when using Caco-2 cells rather than T84 cells (17). A concentration of 100 IU/mL IFN-γ had no effect on the expression or rearrangement of occludin or claudin-1 compared to control monolayers (17). However, translocation of *E. coli* C25 occurred at IFN-γ concentrations greater than or equal to 10 IU/mL in Caco-2 monolayers (17) Overall, the results indicated IFN-γ may induce the translocation of nonpathogenic *E. coli* across an unaltered epithelium without disrupting tight junction integrity, in terms of occludin and claudin-1 expression levels (17).

Lipid rafts may allow a portal of entry for bacteria into eukaryotic cells (17). Therefore, Clark et al. investigated whether IFN-γ-mediated translocation of *E. coli* C25 in Caco-2 cells occurred via raft-dependent pathways (17). The experiment involved the use of filipin and methyl-β-cyclodextrin (MCD), agents that sequester membrane cholesterol and disrupt raft-dependent processes (17). Pre-treatment of Caco-2 cells with filipin and MCD dose-dependently inhibited IFN-γ-mediated translocation of *E. coli* C25 (17).

Clark et al. further investigated the lipid rafts as a means of bacterial translocation with the assessment of the ganglioside GM1, a marker of raft formation that localizes to lipid rafts (17). The Caco-2 cells were incubated with or without 100 IU/mL of IFN-γ labeled with cholera toxin, which specifically binds to GM1 (17). GM1 levels markedly increased after incubation with IFN-γ compared to untreated controls (17).

In conclusion, the results of the study challenge the concept that tight junction disruption is necessary for IFN-γ-mediated translocation of nonpathogenic bacteria (17). It is believed that a loss of tight junction integrity leads to an increase in paracellular permeability, which then allows bacterial translocation followed by a prolonged inflammatory response (17). Clark et al provided contradictory evidence to the previous hypothesis, by demonstrating IFN-γ-exposed T84 and Caco-2 monolayers promote commensal *E. coli* C25 translocation, in the absence of paracellular permeability and tight junction alterations (17). This may be explained by the ability of IFN-γ to upregulate immune accessory molecules, such as integrins, that are associated with attachment and internalization of commensal bacteria (17).

Clark et al. also demonstrated a novel mechanism by which enteric bacteria invade the epithelium: lipid rafts (17). These rafts, or membrane microdomains, contain cholesterol and glycosphingolipids and are involved in many cellular processes (17). Clark et al. showed IFN-γ induced cellular changes allow *E. coli* C25 to use lipid rafts as a means of internalization (17). This process may also enhance the survival of *E. coli* C25, as phagosomes derived from lipid rafts can avoid degradation by the host cell lysosomes (17)

Overall, the findings of the study suggest commensal bacteria may use a lipid raft-mediated pathway, under inflammatory stress, to cross the epithelial barrier (17). As this process was demonstrated at low concentrations of IFN-γ, it may occur prior to the disruption of tight junctions (17). Further studies will be necessary to investigate the cellular changes that take place in order for IFN-γ to allow *E. coli* C25 to gain access to epithelial monolayers, such as cellular signaling processes and IFN-γ-induced phenotypic changes in intestinal cells (17).

2. The Effects of Enteroaggregative *Escherichia coli* (EAEC) vs. Commensal *Escherichia coli* on Tight Junction Protein Localization

Enteroaggregative *Escherichia coli* (EAEC), one of the most common bacterial pathogens occurring in patients with diarrhea, adheres to the intestinal mucosa where it releases enterotoxins (18). The adherence of EAEC to the intestines is mediated by the aggregative adherence fimbriae (AAF), which are related to the adhesions of some enteropathogenic *E. coli* (18). A particular strain of EAEC, strain 042, exhibits the AAF/II variant, which has been implicated in the delocalization of tight junction proteins

and loss of epithelial integrity during infection (18).

Strauman at al. assessed the effects of EAEC strain 042 on intestinal epithelial integrity in comparison to a commensal strain of *E. coli* (18). The results of the study demonstrated AAF/II as a necessity for barrier dysfunction during EAEC infection (18). The authors also compared the effects of EAEC on tight junction protein structure and localization *in vitro* compared to commensal *E. coli* strain HS or 042aafA (18).

First, polarized T84 intestinal epithelial cell monolayers were infected with EAEC strain 042 for 3 hours and TER was measured (18). After the infection period, the monolayers were washed and TER was measured at different intervals for 21 hours (18).

At the end of the 3 hour infection period, there was no difference found in TER between strain 042-infected and uninfected monolayers (18). However, during the hours after the 3 hour infection period, the TER decreased significantly in the strain 042-infected monolayers compared to uninfected monolayers and those infected with commensal *E. coli* strain HS (18).

Next, in order to investigate barrier function directly, Strauman et al. assessed monolayer permeability to FITC-conjugated dextran and FITC-conjugated BSA (18). The ability of these molecules to move across the paracellular space was determined by fluorescence in the basolateral compartment (18). After infection with strain 042, augmented translocation of FITC-dextran and FITC-BSA was observed into the basolateral compartment (18). Strain 042 infection did not enhance FITC-BSA flux; therefore the authors concluded AAF/II was required to increase barrier

permeability (18).

Strauman et al. then ruled out the possibility that EAEC affected TER through a cell death mechanism by using the fluorescent Live/Dead viability/cytotoxicity kit assay (18). The assay was used to quantitate the number of nonviable cells versus viable cells in 042-infected T84 monolayers, where the live cells appear green and the nonviable cells appear red (18). The cells were infected for 3 hours, and the assay was performed 21 hours later (18). No significant difference was found in the number of dead cells in monolayers infected with 042 compared to uninfected and commensal strain HS monolayers (18). Therefore, an EAEC 042 induced decrease in TER is not the result of cell death (18).

Strauman et al. then investigated the effects of EAEC on the tight junction proteins occludin, ZO-1, and claudin-1 (18). Strauman et al. hypothesized that the EAEC induced increase in paracellular permeability was associated with the redistribution of tight junction proteins (18). In uninfected T84 monolayers and T84 monolayers infected with commensal *E. coli* strain HS, peripheral colocalization of occludin and ZO-1 occurred (18). However, in monolayers infected with strain 042, jagged intercellular junctions and dissociation of occludin and ZO-1 within the tight junctions were observed (Figure 9).

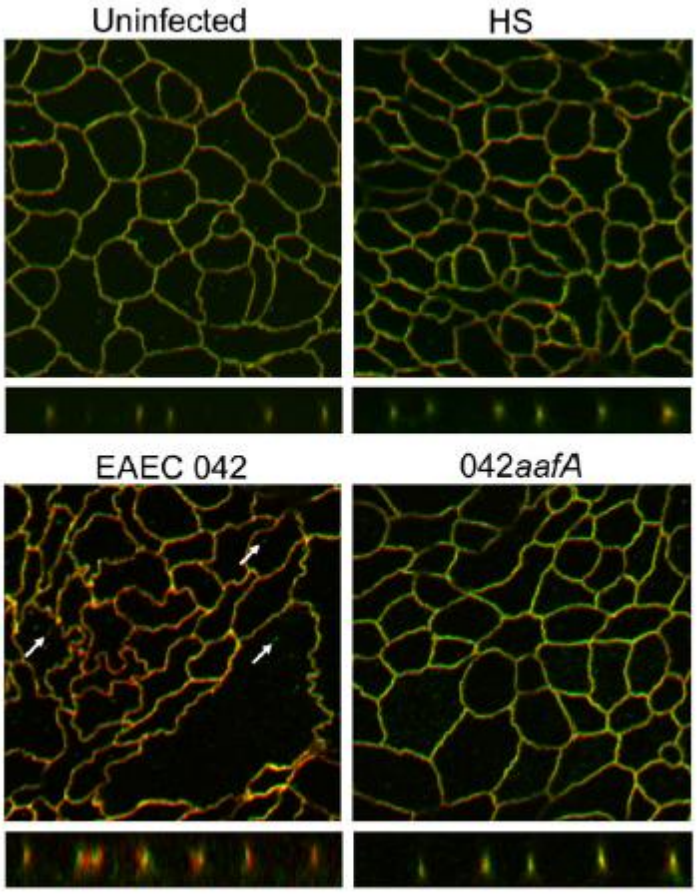

Figure 9. Irregular morphology of T84 membranes and occludin delocalization following infection with EAEC strain 042 and JM221. HS is a commensal *E. coli* strain. Occludin (green) and ZO-1 (red). Adapted from Strauman et al. 2010.

In terms of cellular morphology, large, elongated cells in strain 042 infected monolayers were seen, compared to that observed in uninfected and commensal strain HS infected monolayers. In fact, some cells within the strain 042 monolayers even contained more than one nucleus, as evident with DAPI staining, which may suggest synctium formation (Figure 10). Claudin-1 was also dissociated from the tight junctions in strain 042 infected monolayers; whereas no dissociation of claudin-1 was observed in uninfected and commensal strain HS infected controls (18).

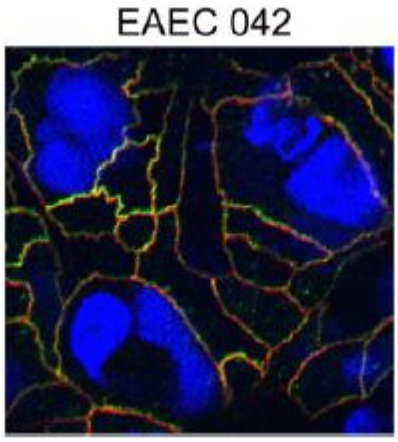

Figure 10. EAEC 042 infection results in the formation of large multinucleate cells. Occludin (green), ZO-1 (red), and nuclei (DAPI). Adapted from Strauman et al. 2010.

In conclusion, Strauman et al. reported a loss of barrier function induced by EAEC infection of T84 polarized monolayers (18). In relation to the previously discussed studies, these experiments involved the comparison of a pathogenic strain of *E. coli* to a nonpathogenic commensal strain of *E. coli*. Strauman et al showed a minimal effect on tight junction proteins caused by commensal *E. coli* in comparison to EAEC (18).

3. Interkingdom Signaling Between Commensal Bacteria and Host Cells

Recently an important discovery was made involving interkingdom signaling between commensal bacteria and host cells in the human GI tract (19). A specific bacterial signal, indole, is recognized as beneficial by intestinal epithelial cells (19). The human GI tract is home to approximately 10^{14} nonpathogenic commensal bacteria that secrete various signaling molecules involved in the regulation of homeostasis and

infections (19). Previous studies have focused on the recognition of human signals by bacteria; however, Bansal et al. investigated the recognition of bacterial signals by human epithelial cells (19).

The bacterial signal indole is produced in *E. coli* via tryptophanase; however, its physiological role is poorly understood (19). It is likely that intestinal epithelial cells are exposed to indole at high concentrations, as *E. coli* produce up to 600 uM of indole in suspension cultures and the same concentration was detected in human feces (19). As Bansal et al. hypothesized indole was beneficial to intestinal epithelial cells, changes in gene expression were assessed with the human enterocyte cell line HCT-8 after exposure to indole (19). Bansal et al. also assessed phenotypic measurements of TER, nuclear factor kappa-B (NF-kB) activation, and IL-8 and IL-10 secretion (19)

The human colon cancer cell line HCT-8 was chosen because it is a polarizable cell line used for investigating mechanisms of host-cell response to pathogens and inflammation (19). HCT-8 cells were exposed to 1 mM indole or solvent for 4 hours or 24 hours (19). Next, RNA isolation and microarrays were performed to identify statistically significant changes in gene expression (19).

Measurement of NF-κB activation was obtained with HCT-8 cells transduced with a NF-κB-GFP reporter lentivirus (in order for the cell line to express NF-κB) and then exposed to indole or indole-like molecules for a period of 4 hours (19). Then 40ng/mL tumor necrosis factor-alpha (TNF-α) was added, and image analysis was performed with fluorescence microscopy (19). Flow cytometry and intracellular cytokine staining were used to assess IL-10 expression in HCT-8 cells using phycoerythrin-

conjugated anti-human IL-10 antibody (19). IL-8 expression was determined with ELISA (19)

Intestinal epithelial cell gene expression was determined with whole-transcriptome profiling (19). Exposure of cells to indole for 4 hours showed differential expression of 523 genes (476 genes induced and 47 repressed) (19). Indole induced specific genes associated with epithelial cell structure and function, including genes responsible for tight junction organization (19). Seven claudin genes were induced after 24 hour exposure to indole, which may indicate an increase in paracellular resistance (19). This observation is further supported by a decrease in pore-forming claudin-2 (cldn2) expression (19).

Several other important proteins were induced by indole; tight junction proteins TJP1, TJP3, and TJP4, which are downstream of claudin-mediated tight junction regulation (19). Indole induced cytoskeleton gene expression as well as a coordinated induction of several cytoskeleton gene families, including actinin and cingulin (19). In addition, several genes responsible for mucin production were induced by indole (19). This is important because an increase in mucin production may weaken pathogen colonization (19).

Next, in order to assess the effect of indole on tight junction function and cell permeability, the TER of HCT-8 epithelial cells was measured after 4 hours and 24 hours exposure to indole (19). The results showed an indole-induced increase in TER within 4 hours and a 1.6 fold increase after 24 hours (19). Also, when HCT-8 cells were pretreated with 1mM indole for 24 hours, there was a decrease in EHEC colonization,

further supporting an indole-induced resistance to pathogen colonization (19). Overall, the results of the gene expression analysis support the idea that indole improves epithelial barrier function and may induce resistance to pathogens within the intestinal epithelium (19).

Bansal et al. also investigated the role of indole in attenuating NF-kB activation and the promotion of other epithelial barrier properties such as TER (19). First, the effects of 5 indole-like molecules, H-indole-2,3-dione (isatin), 7-hydroxyindole (7-HI), 5-hydroxyindole (5-HI), 2-hydroxyindole (2-HI), and indole-3-acetic acid (I3AA), were determined compared to indole (19). Only 5-HI significantly attenuated NF-kB activity similar to indole (19). Isatin actually increased NF-kB activity (19). Only 7-HI demonstrated an increase in TER comparable to indole (19). Overall, the results showed indole-like molecules were not capable of altering both NF-kB activity and TER simultaneously, as indole could (19).

As there is little evidence for recognition of bacterial signals by host cells, Bansal et al. provided insight into interkingdom communication and its role in pathogenesis (19). The bacterial signal indole is recognized by intestinal epithelial cells and is beneficial in terms of improving barrier function and resisting pathogen colonization (19). Indole is produced by several commensal bacteria within the GI tract, including *E. coli*, which may indicate the beneficial effects of commensal bacteria are mediated through indole signaling (19).

The effects of indole on TER, inflammation, and paracellular permeability on intestinal epithelial cells are quite relevant to intestinal inflammatory diseases, such as

Crohn's disease (19). Bansal et al. proposed indole as a novel and safe treatment for such intestinal diseases, as it is naturally present in the GI tract (19). In conclusion, Bansal et al. showed interkingdom signaling between intestinal epithelial cells and commensal bacteria may be beneficial for epithelial integrity (19).

	In vitro/ in vivo	What?	Mechanisms	Implications
Nazli et al. 2004	*In vitro*	Under metabolic stress (DNP), epithelia perceive commensal bacteria as a threat	DNP + E. coli HB101 increase paracellular permeability and decrease TER	May exacerbate IBD during times of stress in host
Nazli et al. 2006	*In vitro*	Under metabolic stress, increased endocytic activity and epithelial permeability lead to increased internalization of bacteria	Decrease in ZO-1 and occludin expression	During stress, increased bacterial translocation into mucosa evoke excessive inflammatory response (IBD)
Clark et al. 2005	*In vitro*	IFN-γ induces an increase in translocation of commensal E. coli across gut epithelial cells	Lipid raft-mediated transcytotic pathways, uncoupled from tight junction protein disruption	During inflammatory stress, normally non invasive commensal bacteria may cross epithelium, IBD
Strauman et al. 2010	*In vitro*	EAEC disrupts epithelial cell tight junctions	Decrease in TER, redistribution of claudin-1 and occludin, mediated by AAF adhesins	EAEC pathogenic mechanisms not fully understood, first report of barrier function loss
Bansal et al. 2010	*In vitro*	Bacterial signal indole is recognized as a beneficial signal in intestinal epithelial cells	Secreted by commensal E. coli, exposure of cells to indole induced increased expression of genes involved in strengthening mucosal barrier	Crohn's disease, possible treatment to regulate intestinal inflammation and promote epithelial cell function

Table 1. A summary of the effects of commensal *Escherichia coli* on tight junction permeability.

IV. THE EFFECTS OF ENTEROPATHOGENIC *ESCHERICHIA COLI* ON TIGHT JUNCTION PERMEABILITY

1. The effects of Enteropathogenic *Escherichia coli* (EPEC) on Occludin and ZO-1 Redistribution

Enteropathogenic *Escherichia coli* (EPEC) is a diarrheal disease responsible for the deaths of several hundred thousand children each year (20). The characteristic attaching/effacing (A/E) lesion is formed when EPEC colonizes the intestinal epithelial surface (20). The intimate attachment of the bacteria to the epithelial cell membrane is believed to play a crucial role in EPEC pathogenicity (20). The pathogenic mechanism utilized by EPEC consists of effector virulence proteins injected into host cells via the type three secretion system (TTSS), which is encoded by a pathogenicity island known as the locus of enterocyte effacement (LEE) (20).

The EPEC secreted effector protein F (EspF) is necessary for disruption of tight junction barrier function *in vitro* (20). Studies using epithelial cell lines in tissue culture have demonstrated EPEC infection leads to a decrease in TER or polarized epithelial monolayers as well as a disruption of tight junction barrier function through redistribution, dephosphorylation, and dissociation of tight junction proteins (20). Zhang et al. investigated the effects of EPEC infection on tight junction barrier function *in vivo* (20).

Zhang et al. used 4 to 6-week old male C57BL/6J mice as an *in vivo* model of EPEC infection (20). The mice were infected with wild-type (WT) EPEC and a mutant EPEC strain that was missing the EspF gene (ΔespF) by oral gavage of 2×10^8 EPEC suspended in 200 ul of sterile PBS (20). The control element consisted of 200 ul of sterile PBS (20). For the histological analysis, colon tissues of control and EPEC infected mice were excised, washed with PBS, and fixed in formalin (20). Tissue sections were cut with a microtome and stained with hemetoxylin and eosin (H&E) (20).

Sections of colon were also excised for transmission electron microscopy analysis (20). The tissue sections were fixed in gluteraldehyde, post fixed in OsO_4, dehydrated through graded alcohols, infiltrated through Epon 812, and then embedded in resin (20). Next, sections were cut and stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (20).

Tissues used in immunofluorescence analysis were incubated with monoclonal antibodies against occludin or ZO-1, washed, then incubated with secondary antibodies (20). The images were examined with a laser confocal scanning microscope (20).

Mucosal permeability was also assessed using a tracer experiment (20). After the mice were sacrificed, biotin was injected into the colon (20). The portion of the colon in contact with the biotin solution was cut, preserved, washed, sectioned, and then examined using a confocal scanning microscope (20).

Upon microscopic examination of the dissected colons from the mice, the results indicated well formed stools in the colons of the control mice and engorged colons with diffuse stools in the EPEC infected mice (20). The EPEC infected mice also showed mild intestinal inflammation of the ileum 5 days post infection (20). Pathological changes in the colonic mucosa of EPEC infected mice were more severe on day five compared to control mice (20).

Next, Zhang et al. investigated changes in tight junction ultrastructure in EPEC infected mice (20). In the control mice, tight junction morphology appeared typical with intact membrane fusions and desmosomes (Figure 11). However, in EPEC infected mice,

tight junctions were discontinuous with decreased membrane fusions (Figure 11). The mice infected with the mutant strain (ΔespF) of EPEC displayed similar tight junction morphology compared to control mice 1 day post infection (Figure 11). At 5 days post infection, the mice infected with the mutant EPEC strain displayed altered tight junction morphology, as well as a disappeared desmosome (Figure 11).

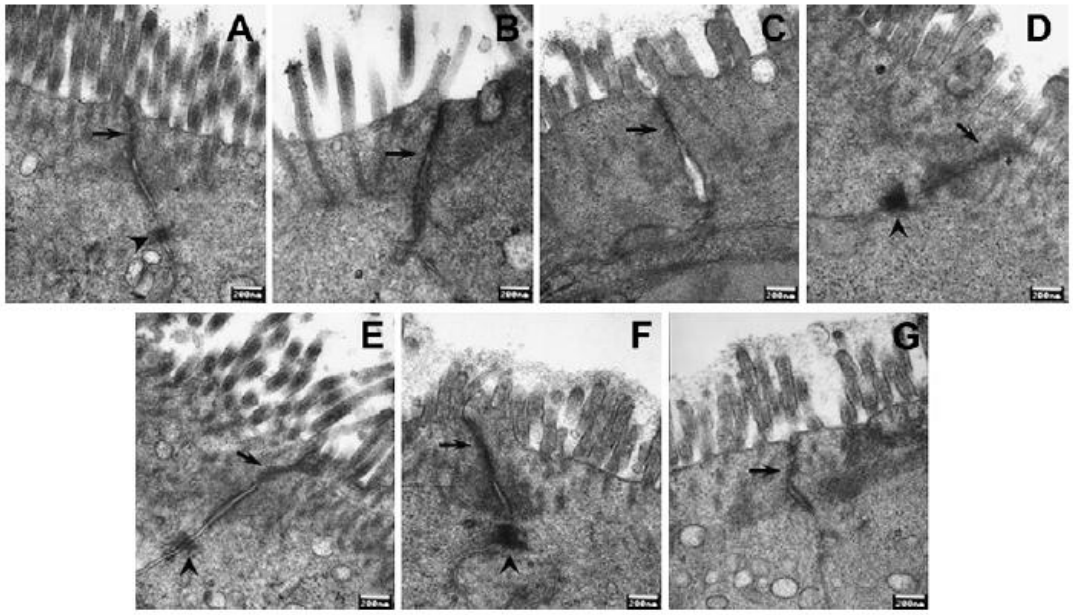

Figure 11. Alteration of tight junction morphology after 5 days infection with EPEC. Intact tight junction structure and normal desmosomes in control mice (A) WT EPEC infected mice, abnormal tight junctions and desmosomes (B-D). Mice infected with ΔespF for 1, 3, and 5 days (E-G). Arrows: tight junction, arrow heads: desmosomes. Adapted from Zhang et al. 2010.

Zhang et al. then examined whether tight junction barrier function was altered during EPEC infection with the tracer experiment (20). In the control mice, the biotin fluorescent signals were restricted to the lumen of the colon (20). In EPEC infected

mice, the biotin permeated the epithelium into the lamina propria and was distributed diffusely in the colon tissue (20). In the mutant EPEC infected mice, the tight junctions appeared to be intact up until 5 days post infection, when the biotin tracer penetrated into the tissue (20).

Next, the localization of the tight junction proteins occludin and ZO-1 during EPEC infection were assessed with immunofluorescence experiments (Figure 12). During EPEC infection, occludin was redistributed to the apical region of epithelial cells,

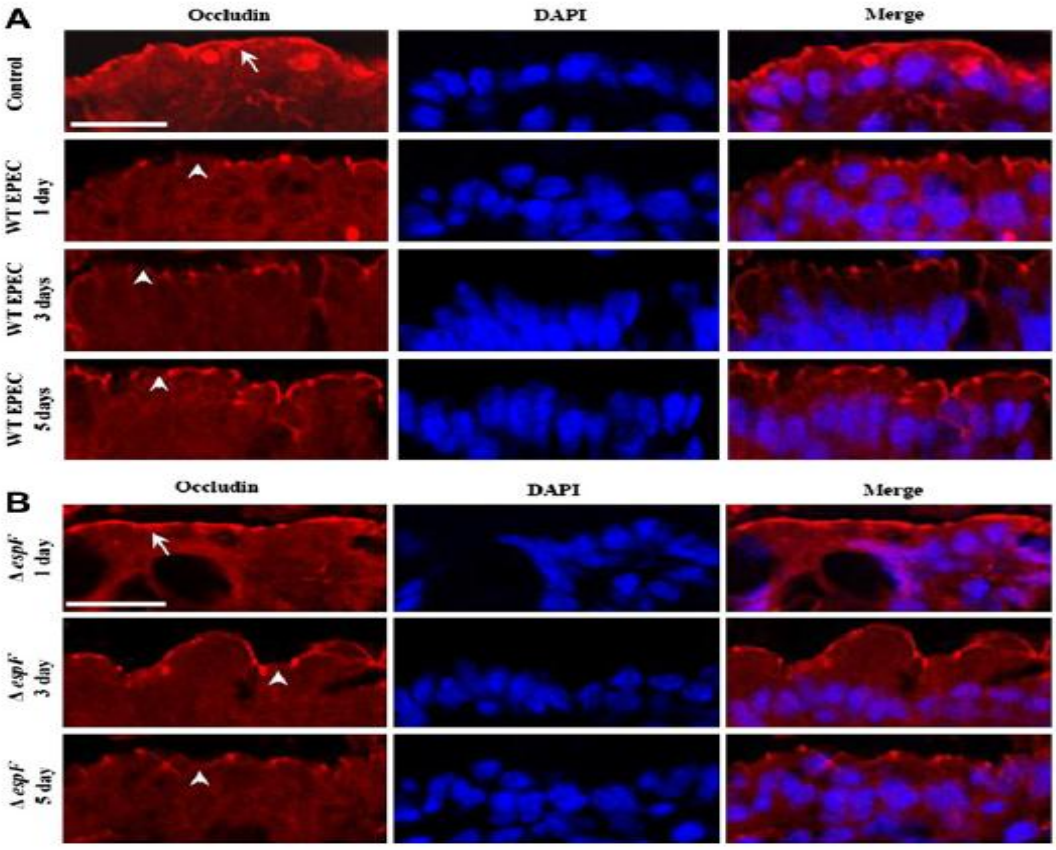

Figure 12. The redistribution of occludin in EPEC infected mice. Arrows: localization of tight junction proteins at tight junctions, arrowheads: lack of tight junction protein staining. Adapted from Zhang et al. 2010.

compared to control mice (20). In mice infected with the mutant strain ΔespF, occludin distribution was the same as control mice up until 5 days post infection, where it was present in the tight junction and cytoplasm (20).

The distribution of ZO-1 was also altered during EPEC infection (Figure 13). ZO-1 was localized diffusely within cells infected with EPEC (20). In control mice, ZO-1 remained in the epithelial cell membrane (20). A change in ZO-1 distribution was not

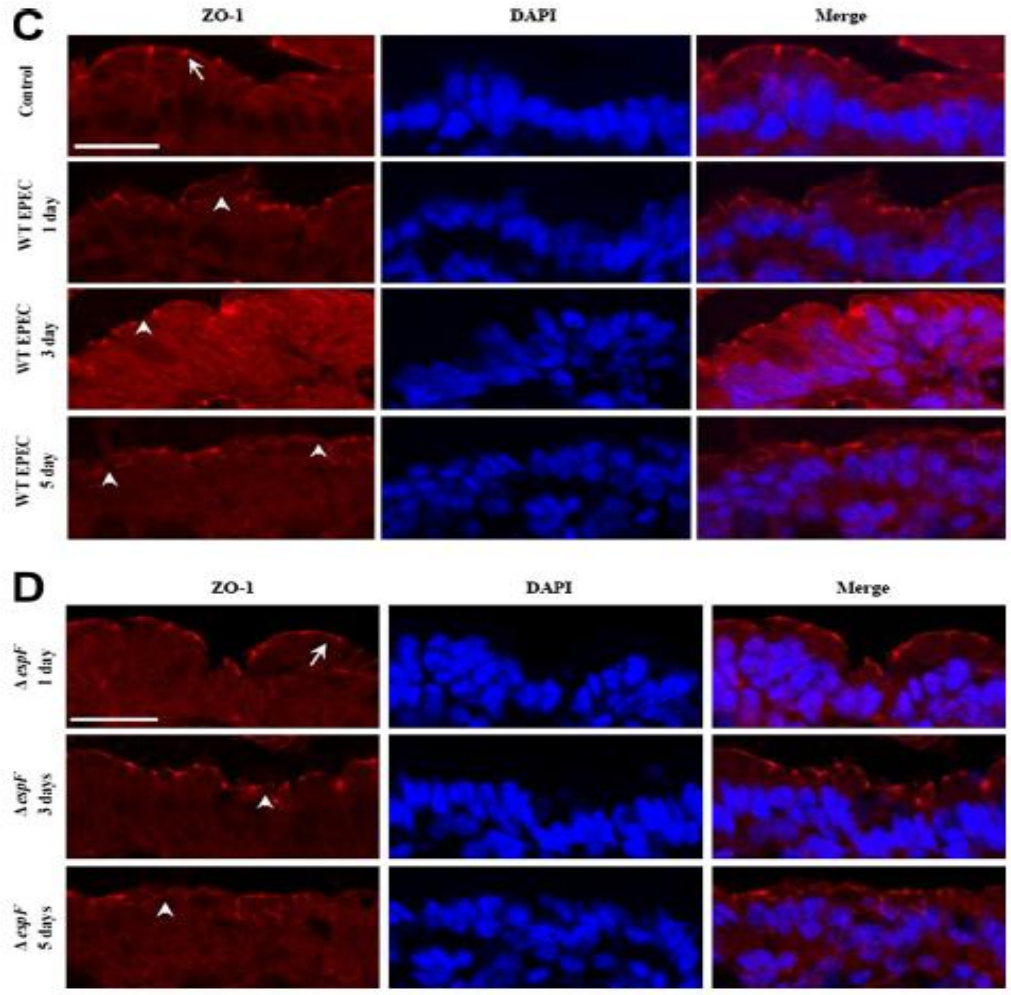

Figure 13. The redistribution of ZO-1 in EPEC infected mice. Arrows: localization of tight junction proteins at tight junctions, arrowheads: lack of tight junction protein staining. Adapted from Zhang et al. 2010.

observed in mice infected with the mutant strain of EPEC until 5 days post infection (20).

Overall the results of the study demonstrated an EPEC-induced disruption of tight junctions caused by an increase in permeability and alteration of tight junction structure (20). The disruption in tight junction function was evident by the redistribution of occludin and ZO-1 in the colon tissues (20). The study is of importance because very little is understood about the *in vivo* effects of EPEC on tight junction permeability (20).

As EspF plays a major role in the pathogenesis of EPEC, Zhang et al. included a mutant strain of EPEC (ΔespF) in the experiments (20). Mice infected with the mutant strain of EPEC showed little change in intestinal barrier function at 1 day post infection, suggesting EspF is necessary for altered barrier function during EPEC infection (20). In conclusion, Zhang et al. shed light on the mechanism by which EPEC disrupts tight junction barrier function *in vivo*, which included a redistribution of the tight junction proteins occludin and ZO-1 (20).

During infection with EPEC, host cytoskeletal proteins are phosphorylated beneath the formed lesions (21). The light chain of myosin (MLC) is the main protein that is phosphorylated during EPEC infection and is involved in the regulation of tight junction permeability (21). The tight junction protein occludin also requires phosphorylation in order to remain associated with the membrane at the level of the tight junction (21).

Phosphorylated occludin is also believed to play a major role in forming the tight junction "seal" (21). Simonovic et al. investigated the effects of EPEC on the tight junction protein occludin in comparison to non-pathogenic *E. coli* (21).

Dephosphorylation of occludin may contribute to the pathogenicity of EPEC (21).

First, human T84 intestinal epithelial cells were infected with EPEC for 1, 3, or 5 hours before immunostaining with occludin (21). After only one hour post infection, occludin was still confined to the membrane; however, distribution took on a beaded appearance (Figure 14). After 3 hours of infection occludin became redistributed to an intracellular compartment and showed a decreased association with tight junctions compared to uninfected monolayers (Figure 14).

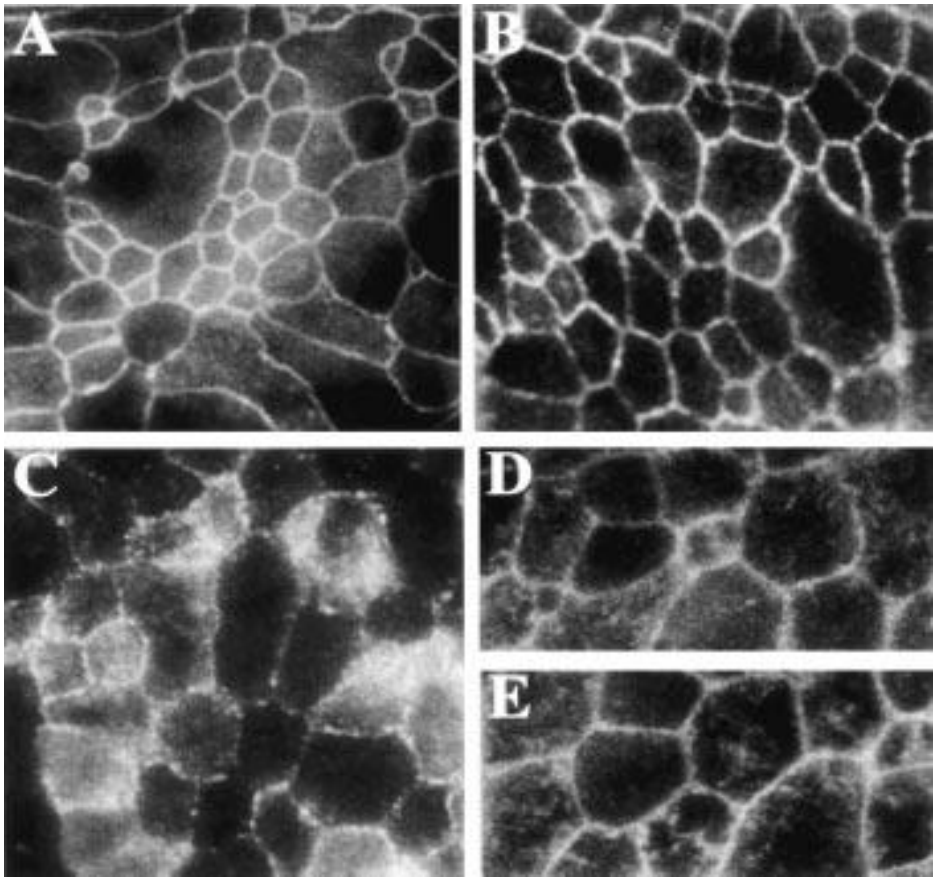

Figure 14. The redistribution of occludin in EPEC infected cells. (A). Uninfected control cells demonstrate localization of occludin. (B). Occludin distribution takes on a beaded appearance after 1 hour of infection with EPEC. (C). Dissociation of occludin from the membrane after 3 hours of EPEC infection. (D and E). Actin distribution within control (D) and EPEC infected cells (E) is unchanged. Adapted from Simonovic et al. 2000.

When separated by SDS-PAGE and identified by immunoblotting, occludin appears as two distinct bands, the higher molecular weight band being hyperphosphorylated occludin (21). Hyperphosphorylated occludin localizes to the tight junction (21). The lower molecular weight band, or non-phosphorylated occludin, is located within a cytoplasmic compartment and/or the basolateral membrane (21).

Simonovic et al. then investigated the effects of EPEC on occludin phosphorylation in T84 cells compared to uninfected controls (Figure 15). In the uninfected control cells, occludin mostly resolved within the high molecular weight band, which indicates hyperphosphorylation (Figure 15). In EPEC infected cells, the results showed a reciprocal relationship in accordance with time (Figure 15). Therefore, it can be inferred that EPEC infection in epithelial cells leads to dephosphorylation of occludin, thereby disassociating the protein from the tight junction (21).

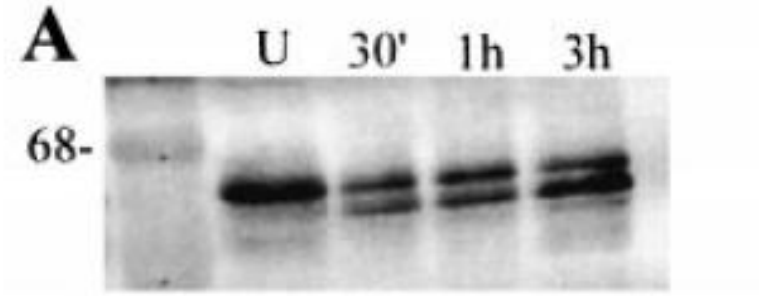

Figure 15. EPEC infection of intestinal T84 cells induces dephosphorylation of occludin. The first lane, U, represents uninfected control cells. The following lanes represent 30 min, 1 hour, and 3 hours post infection with EPEC. The upper band represents hyperphosphorylated occludin and the lower band represents unphosphorylated occludin (19). Adapted from Simonovic et al. 2000.

Next, Simonovic et al investigated whether the serine/threonine phosphatase inhibitor, calyculin A, would prevent the redistribution of occludin and decrease in TER caused by EPEC infection (21). The inhibitor was chosen because occludin is phosphorylated on both serine and threonine residues (21).

The T84 monolayers were infected with EPEC in the absence or presence of calyculin A (3 and 4nM) and examined with immunofluorescence microscopy (21). Calyculin A (at both concentrations) fully prevented the dissociation of occludin from the tight junctions in the EPEC infected monolayers (21).

Expression of certain genes within the locus of enterocyte effacement (LEE) plays a role in host cell physiology during EPEC infection (21). In fact, when non-pathogenic *E. coli* K-12 is transformed with LEE of EPEC, *E. coli* K-12 is able to form A/E lesions and decrease TER (21). Therefore, Simonovic et al. investigated the effects of non-pathogenic *E. coli* K-12 and a LEE-transformed K-12 on occludin distribution (21).

E. coli K-12 had no effect on occludin localization (Figure 16).

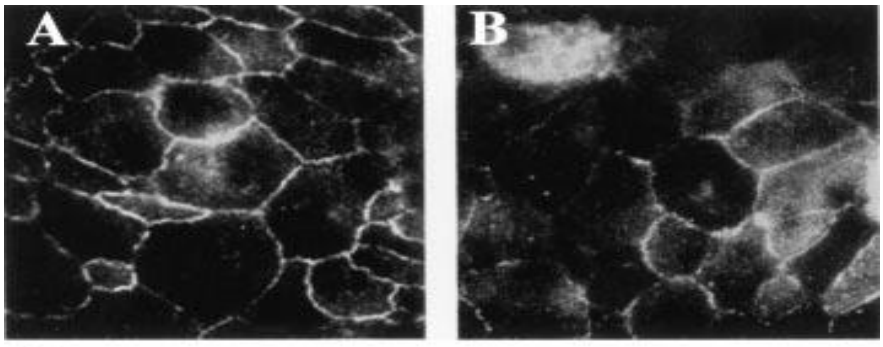

Figure 16. Occludin redistribution in T84 cells infected with non-pathogenic *E. coli* K-12 (A) and *E. coli* K-12 transformed with LEE (B) for 3 hours. Adapted from Simonovic et al. 2000.

However, the LEE-transformed K-12 displayed occludin localization identical to that of wild-type EPEC, and a decrease in TER very similar to wild-type EPEC (21).

Overall, EPEC induced phosphorylation of the tight junction protein occludin, which leads to the dissociation of occludin from the tight junction (21). These effects were also prevented with the serine-threonine phosphatase inhibitor calyculin A (21). Interestingly, when non-pathogenic *E. coli* K-12 is transformed with the LEE of EPEC, it takes on the phenotype of wild-type EPEC (21).

Simonovic et al. discussed how the integrity of the tight junction is not dependent upon a single protein, such as occludin (21). Therefore, future studies should focus on the effects of EPEC on each tight junction protein involved in barrier function, such as the claudin family and ZO-1 (21). Future experiments should examine whether the EPEC induced dephosphorylation of occludin also induces other effects on claudin or ZO-1 in order to fully disrupt barrier function.

As discussed previously, the EPEC effector protein EspF is believed to play a crucial role in decreasing TER and altering tight junction structure in the intestinal epithelium (22). However, the mechanism by which EspF disrupts barrier function *in vitro* has not been fully explained (22). As small animal models for studying EPEC *in vivo* are limited, Shifflett et al. set out to establish the C57BL/6J mouse as a suitable model for EPEC infection in order to investigate the effects of EPEC on tight junctions (22). Shifflett et al. also included an examination of the role of EspF in the mouse model (22).

The experimental methods included male 6 week old C57BL/6J mice that were

gavaged with 200 uL of sterile PBS as a control or 2 x 10^8 EPEC suspended in 200 uL sterile PBS (22). The mice were killed by asphyxiation at 1 or 5 days post infection and intestinal tissue was resected (22). Ileal and colonic tissue were mounted in Ussing chambers to measure TER (22). Ileal and colonic tissues were also snap-frozen and analyzed with immunofluorescent microscopy (22).

Attachment assays were performed in order to assess the level of attachment of wild-type (WT) EPEC and mutant EPEC strains to the ileum and colon of the mice (22). Ileal and colonic tissues were also snap frozen in liquid nitrogen immediately after euthanasia for protein analysis with gel electrophoresis and western blotting (22). RNA was extracted from ileal and colonic tissues and analyzed with quantitative real-time PCR (RT-PCR) (22).

The results indicated EPEC colonization of the ileum occurred 1 day post infection and continued for 5 days post infection (22). Furthermore, WT EPEC and ΔespF showed the same level of colonization after 1 and 5 days post infection (22). WT EPEC significantly reduced the barrier function of the ileum and the colon, as determined by TER measurement (22). However, at 1 day post infection the ΔespF strain showed no effect on the barrier function of the ileum and colon (22).

Next, Schifflett et al. examined whether the WT EPEC-induced change in barrier function was a result of a change in tight junction structure (22). As expected, occludin was found at the tight junction and dissociated to the apical and basal cytoplasm of ileal and colonic epithelial cells of WT EPEC infected mice at day 1 (22). Occludin remained localized at the tight junctions in the ileum and colon of the uninfected control mice as

well as ΔespF infected mice at day 1 (22).

The tight junction protein ZO-1 was localized with the perijunctional actomyosin ring in control uninfected mice and ΔespF infected mice (22). Interestingly, ZO-1 was also localized to the tight junction in EPEC-infected mice (22). Therefore, the redistribution of tight junction proteins during EPEC infection may not be a global effect (22).

After 5 days post infection, the effect of the WT EPEC infected mice on barrier function was similar to that of 1 day post infection (22). In ΔespF infected mice the barrier function of the ileum and colon was at the same level induced by WT EPEC at 5 days post infection (22). Also, in ΔespF infected mice, the distribution of occludin and ZO-1 in ileal and colonic epithelium were indistinguishable from that of WT EPEC infected mice at 5 days post infection (22). Therefore, barrier function in ΔespF infected mice is preserved at 1 day post infection and disrupted at 5 days post infection (22).

Overall, Schifflett et al. demonstrated EPEC infection disrupts intestinal barrier function *in vivo* (22). Specifically, EPEC induces a redistribution of the tight junction protein occludin (22). The alterations in barrier function are mediated by EspF during early infection (1 day post infection) but not at later time points (5 days post infection) (22).

It is interesting to note however, the results obtained by Schifflett et al. are different from the previously discussed study by Zhang et al. (20, 22). Zhang et al. demonstrated changes in the distribution of the tight junction protein ZO-1 in WT EPEC infected mice as well as ΔespF infected mice (20). The changes were also time

dependent, as they occurred 5 days post infection (20). Schifflett et al. reported no change in the distribution of ZO-1; only changes in the distribution of occludin were observed (22).

In both studies, similar mice models were used, as well as similar experimental methods (20, 22). Future studies should aim to rectify the contrasting results, in order to verify whether the tight junction protein ZO-1 is actually redistributed during infection with EPEC by assessing ZO-1 localization and expression level at 1, 3, and 5 days post infection with EPEC. This information is important as ZO-1 is crucial to tight junction integrity and barrier function.

2. Type Three Secretion System Effector Proteins

In addition to EspF, there are other effector molecules delivered into the host cell that alter epithelial paracellular permeability during EPEC infection, such as EspG and EspG2 (21). Matsuzawa et al. examined the effects of the type III effectors EspG and EspG2 on epithelial paracellular permeability and tight junction architecture in MDCK monolayer cells (23).

Matsuzawa et al. first confirmed EspG2 was secreted via the TTSS by preparing secreted proteins from bacterial culture supernatant, followed by a Western blot analysis using anti-EspG2 antibodies (23). Next, Matsuzawa et al. confirmed EPEC injects EspG2 into the host cell via the TTSS using a fluorescence-based reporter system (23). Plasmids encoding TEM-1 fused proteins were introduced into EPEC, thereby demonstrating the secretion of TEM-1-fused proteins (23).

The EPEC secreted effector proteins EspG and EspG2, which have been shown to disrupt the host cell microtubule network, can activate the GEF-H1-mediated RhoA-Rho Kinase (ROCK) signaling pathway in HeLa cells (23). This is important because the Rho family of GTPases have been implicated in the maintenance of tight junction assembly and function via the actin cytoskeleton (23). Matsuzawa et al. used an infection assay with EPEC-infected polarized MDCK cells to determine whether RhoA (a central regulator of the actin cytoskeleton) was activated (23). RhoA levels were higher (a 1.7 fold increase) in WT EPEC infected cells as opposed to cells infected with the espG/espG2 double knockout mutant (23).

Next, polarized MDCK cells infected with WT EPEC or the espG/espG2 double knockout mutant were assessed for induction of tight junction disruption (23). A decrease in TER was observed in WT EPEC infected monolayers as well as in the espG/espG2 mutant infected monolayers (23). Furthermore, ZO-1 disruption was observed in both the WT EPEC infected monolayers and the espG/espG2 knockout infected monolayers (23).

Neither EspG or EspG2 induced the distribution of ZO-1 and claudin-1 polarized MDCK cells (23). Next, paracellular permeability was measured in MDCK monolayers expressing EspG or EspG2, in order to determine the effector proteins' involvement in tight junction barrier function (23). In cells expressing EspG or EspG2 there was a five-fold upregulation of the paracellular permeability to 4-kDa FITC-dextran (23). However, the upregulation was not observed in 500-kDa FITC-dextran (23).

Matsuzawa et al. provided evidence for the secretion and translocation of EspG2

into host cells via the TTSS during EPEC infection (23). The activation of RhoA is also dependent upon EspG and EspG2 activity in MDCK cells during EPEC infection (23). However, although EspG and EspG2 were found to alter paracellular permeability, the effectors were not involved in tight junction disruption (23).

As there are many known effectors secreted into host cells via the TTSS during EPEC infection, it will be important to investigate the synergistic effects that take place during pathogenisis (23). The previously discussed effector EspF is still not fully understood; therefore it will also be important to verify the results of the study by Matsuzawa et al. in order to avoid confusion regarding the effects of EspG and EspG2.

Future studies should also assess how EspG, EspG2, and EspF work in concert with one another and if EspG and EspG2 truly have no affect on tight junction function, by evaluating which signaling pathways are activated during effector protein secretion. The experiments should also include an analysis of all crucial tight junction proteins such as ZO-1, occludin, and claudin.

3. The Redistribution of Claudin and Occludin in Tight Junction Membrane Microdomains

Recently, Zhang et al. investigated the effects of EPEC on tight junction permeability *in vivo* (24). Zhang et al. used an A/E mouse infection model to demonstrate the disruption of specific tight junction proteins, occludin and claudin-1, during EPEC pathogenesis (24).

Four- to 6-week-old male C57BL/6J mice were orally infected with WT EPEC

2348/69 (2 x 10^8) EPEC suspended in 200 uL sterile PBS) for 1, 3, or 5 days before they were sacrificed (24). A subset of mice received only sterile PBS as a control (24). Next, the colon tissues of the mice were fixed in formalin, stained with H&E, sectioned, and observed with light microscopy (24).

Zhang et al. also isolated tight junction microdomains using sucrose density gradient centrifugation and homogenizing tissue samples in lysis buffer and protease inhibitor mixture solution, then examined by immunoblotting (24). In order to assess intestinal mucosal permeability, an EZ-link Sulfo-NHS-Biotin tracer was used to visualize penetration of the epithelia (24). The results, as observed with H&E staining, indicated the colon from the control mice was intact and well-organized (24). In the EPEC infected mice, the number of epithelial cells decreased and there was an irregular epithelial surface, mucosal hyperplasia, and infiltration of inflammatory cells (24).

Next, Zhang et al. investigated tight junction protein expression within membrane microdomains of tight junctions (24). The experiment was based on previous reports that the spacial organization of tight junctions is mediated by lipid raft-like compartments (24, 25). Detergent-resistant tight junction membrane microdomains were isolated with detergent extraction and sucrose density gradient centrifugation (24). Proteins were analyzed with Western blotting (24).

EPEC infection induced the redistribution of occludin and claudin-1 out of tight junction membrane microdomains (Figure 17). In the control mice, 19.6% occludin (percentages are measured as a fraction of the density gradient) was observed in tight junction membrane microdomains (24). However, the results showed occludin was

displaced from the low-density fractions to the bottom of the gradient (Figure 17). The tight junction protein claudin-1 was also redistributed in a similar manner (Figure 17).

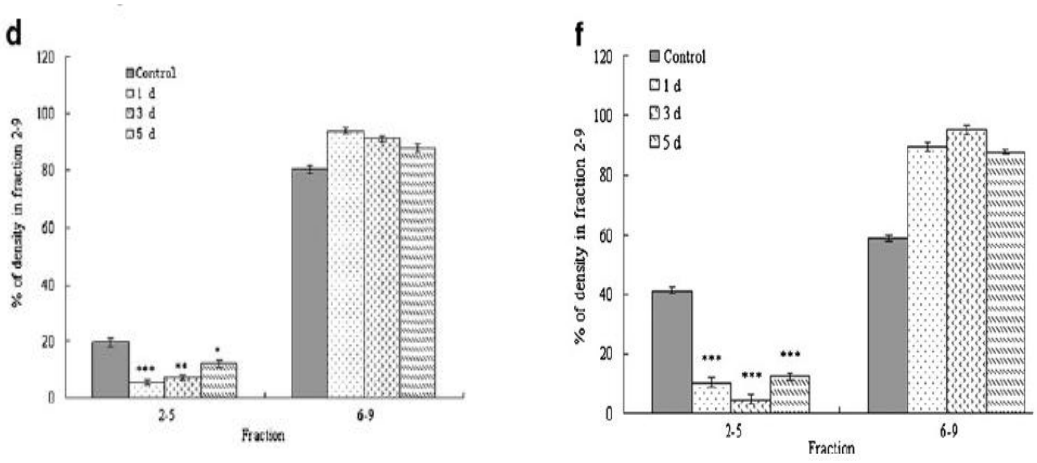

Figure 17. A representation of Western Blott analysis of the distribution of occludin (d) and claudin-1 (f) in tight junction membrane microdomains. Asterisks indicate a significant difference compared to control (*P<0.05, **P<0.01, ***P<0.001). Adapted from Zhang et al. 2012.

The next experiment involved the use of molecular biotin tracer to assess the integrity of the epithelial barrier (24). Claudin-3 and -5 were double-labeled with the biotin tracer (24). In the control model, biotin was restricted to the luminal boundary of the colon epithelium (24). However, once infected with EPEC, the biotin tracer permeated the epithelium and entered into the lamina propria (24). The results showed the tracer entered into the epithelium through areas of altered claudin location (24).

Overall, tight junctions were functionally altered by EPEC infection *in vivo* (24). The changes in tight junction function were associated with a redistribution of occludin and claudin in tight junction membrane microdomains (24). The results are of clinical

relevance as claudin expression was altered in patients with Crohn's disease (24).

Zhang et al. also expanded upon the previously discussed study, which included several of the same authors, by investigating the tight junction protein claudin (20, 24). Previously, Zhang et al. used a biotin tracer and immunofluorescent microscopy to assess paracellular permeability and the redistribution of ZO-1 and occludin (20). The results from both studies were similar, in that EPEC infection lead to the redistribution of tight junction proteins *in vivo*, thereby providing further evidence for a loss of tight junction function during EPEC infection (20, 24).

	In vitro/ in vivo	What?	Mechanisms	Implications
Simonovic et al. 2000	*In vitro*	EPEC dephosphorylates occludin, dissociation from TJ	Events correlate temporally with perturbation of TJ permeability	May be involved in pathogenicity
Schifflett et al. 2005	*In vivo*	EPEC disrupts epithelial barrier function	Occludin redistribution, mediated by EspF at early time points of infection (unknown)	Redistribution of TJ proteins is EspF dependent
Zhang et al. 2010	*In vivo*	EPEC induced redistribution of ZO-1 and occludin, correlates with loss of barrier function	Loss of barrier function requires EspF at initial stages of infection	Redistribution of TJ proteins is EspF dependent
Matsuzawa et al. 2005	*In vitro*	EspG/EspG2 alter epithelial paracellular permeability	Not involved in disruption of TJ protein architecture (claudin-1 and ZO-1), induces Rho activation	Rho GTPases central regulators in actin cytoskeleton. Paracellular permeability and TJ disruption during EPEC infection independently regulated by EspG/G2 and EspF
Zhang et al. 2012	*In vivo*	EPEC disrupts TJ barrier function	Redistribution of occludin and claudin from TJ membrane microdomains	Diarrheal pathogenesis of EPEC in vivo, redistribution of TJ proteins plays important role in EPEC induced disruption of epithelial barrier

Table 2. A summary of the effects of enteropathogenic *Escherichia coli* on tight junction permeability.

V. DISCUSSION

The effects of pathogenic and commensal bacteria on host intestinal epithelial cells are not fully understood. The intestinal epithelium has been described as a haven for "cross talk" between the host and its prokaryotic inhabitants, where countless signaling pathways lead to physiological transformations (3). One of the major ways by which bacteria induce physiological changes in the intestinal epithelium is through alteration of tight junctions.

As discussed here, infection with Enteropathogenic *Escherichia coli* (EPEC) is widely associated with the disruption of tight junction proteins (20, 21, 22, 23, and 24). Both *in vitro* and *in vivo* studies have demonstrated EPEC infection induces redistribution of the tight junction proteins occludin, claudin-1, and ZO-1 (20, 22, 23, and 24). It is not yet determined whether the effector proteins secreted by EPEC into the host cell are involved in the alteration of tight junction function. Matsuzawa et al. showed the effector proteins EspG and EspG2 do not play a role in tight junction disruption (23).

However, the EPEC effector protein EspF is widely attributed to alterations in tight junction barrier function (18). A deletion of EspF significantly weakened the effect of EPEC on TER as well as induced alterations in tight junction protein distribution 10). EspF is also required for tight junction disruption during infection with other pathogenic bacteria, such as Enterohemorrhagic *Escherichia coli* and *Citrobacter rodentium* 10). As these pathogens also utilize the production of A/E lesions, this may suggest an evolutionary conserved phenotype (10)

Another effector protein secreted by the TTSS during EPEC infection, NleA, has been implicated in altering epithelial barrier function (26). For example, Thanabalasuriar et al. showed polarized epithelial cells infected with EPEC displayed a NleA-induced dislocation of ZO-1 and occludin, which was independent of NleA's PDZ-binding domain (26).

If EPEC are a non-invasive bacteria and utilize a secretion system for virulence, why would EPEC want to disrupt tight junction barrier function? The intestinal epithelium must maintain a tight barrier between cells in order to regulate the passage of specific nutrients between the digestive tract and the submucosa (10). The transmembrane proteins of tight junctions prevent the unregulated movement of the nutrients between said compartments (10). Therefore, it would be beneficial for EPEC to alter tight junctions during infection, in order to have access to nutrients and continue colonization within the host.

Mucus production in the gut also plays an important role in nutrient availability for microorganisms (27). The secretion of mucus by goblet cells is a defense mechanism against pathogens entering the epithelium (27). Through peristaltic movement, mucins are able to entrap and remove microbes (27). However, mucin also provides a direct source of carbohydrates and peptides to bacteria, enabling their colonization within the host (27).

Recently, commensal bacteria were implicated in the pathogenesis of intestinal diseases, such as IBD, and commensal *E. coli* may alter tight junction barrier function (6). As discussed here, various other factors may contribute to the effects of commensal

E. coli on tight junction permeability, such as metabolic stress (15, 16). According to Nazli et al., epithelial cells under metabolic stress (represented by the chemical stressor DNP) may perceive commensal bacteria as a threat (15).

However, there were several limitations within the studies involving the use of DNP as a chemical stressor. The use of DNP may not accurately mimic physiological conditions during times of stress within the host. Furthermore, the optimal concentration of DNP necessary to "stress" the epithelium was not discussed by Nazli et al. (15). A future study should involve the identification of a physiologically relevant stressor of the host epithelium and determine an appropriate concentration to use in order for the epithelium to perceive commensal bacteria as a threat.

As the effects of commensal *E. coli* on tight junction permeability are not as clear as that of EPEC, future studies should focus on elucidating the potential risks of commensal bacteria on epithelial integrity. In order to determine whether commensal *E. coli* are actually posing as a threat during metabolic stress within the host, the cell signaling pathways induced by commensal *E. coli* must be assessed. Is there a particular effector protein secreted by commensal *E. coli*, similar to that of EPEC, that causes a phenotypic switch during times of stress?

First, a group of human colonic T84 epithelial cells should be treated with a non-pathogenic commensal *E. coli* strain and a chemical stressor. In order to determine which signaling pathways are activated by non-pathogenic commensal *E. coli*, a kinase activity assay will be performed. This would determine which downstream effectors are responsible for the altered barrier function induced by commensal *E. coli* during

metabolic stress.

There is evidence that some commensal bacteria utilize a secretion system, similar to some enteropathogenic bacteria (28). For example, genomic analysis of microbiota in the gut has demonstrated the existence of type III secretion systems in bacterial species that were known to be commensal (28). Whether these secretion systems are necessary for commensal bacteria to become pathogenic is not known (28).

In conclusion, the effects of commensal *E. coli* and EPEC on tight junction permeability range from negligible to severe, indicating the need for more extensive research in the field. As both commensal *E. coli* and EPEC have been implicated in chronic intestinal diseases, it will also be important to determine how alterations in epithelial barrier function can be remedied for clinical applications.

REFERENCES

1. Derikx JPM, Luyer MDP, Heineman E, and WA Buurman. Non-invasive markers of gut wall integrity in health and disease. World Journal of Gastroenterology 2010; 16(42): 5272-5279.

2. Grootjans J, Geertje T, Verdam F, Derikx JPM, Lenaerts K, and WA Buurman. Non-invasive assessment of barrier integrity and function of the human gut. World Journal of Gastrointestinal Surgery 2010; 2(3): 61-69.

3. Berkes J, Viswanathan, Savkovic SD, and G Hecht. Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. Gut 2003; 52: 439-451.

4. Sears CL. Molecular physiology and pathophysiology of tight junctions v. assault of the tight junction by enteric pathogens. American Journal of Physiology Gastrointestinal and Liver Physiology 2000; 279: G1129-G1134.

5. Chen HD, and G Frankel. Enteropathogenic *Escherichia coli*: unraveling pathogenesis. Federation of European Microbiological Societies Microbiology Reviews 2004; 29(2005): 83-98.

6. Packey CD, and RB Sartor. Interplay of commensal and pathogenic bacteria, genetic mutations, and immunoregulatory defects in the pathogenesis of inflammatory bowel disease. Journal of Internal Medicine 2008; 263: 597-606.

7. Hansson GC. Role of mucus layers in gut infection and inflammation. Current Opinion

in Microbiology 2012; 15:57-62.

8. Fava F, and Danese D. Intestinal microbiota in inflammatory bowel disease: friend or foe? World Journal of Gastroenterology 2011; 17(5): 557-566.

9. Franchi L, Kamada N, Nakamura Y, Burberry A, Kuffa P, Suzuki S, Shaw MH, Kim Y, and G Nunez. Nature Immunology 2012; 13:5.

10. Weflen AW, Alto NM, and GA Hecht. Tight junctions and enteropathogenic *E. coli*. Annals of the New York Academy of Sciences. 2009; 1165: 169-174.

11. Guttman JA, and BB Finlay. Tight junctions as targets of infectious agents. Biochemica et Biophysica Acta 2008; 1788(2009): 832-841.

12. Furuse M. Molecular basis of the core structure of tight junctions. Cold Springs Harbor Perspectives in Biology 2010; 2:a002907.

13. Chiba H, Osanai M, Murata M, Kojima T, and N Sawada. Transmembrane proteins of tight junctions. Biochemica et Biophysica Acta 2007; 1778(2008); 588-600.

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

15. Nazli A, Yang P, Jury J, Howe K, Watson JL, Soderholm JD, Sherman PM, Perdue MH, and DM Mckay. Epithelia under metabolic stress perceive commensal bacteria as a threat. American Journal of Pathology 2004; 164(3).

16. Nazli A, Wang A, Steen O, Prescott D, Lu J, Perdue MH, Soderholm JD, Sherman PM, and DM McKay. Enterocyte cytoskeleton changes are crucial for

enhanced translocation of nonpathogenic *Escherichia coli* across metabolically stressed gut epithelia. Infection and Immunity 2006; 74(1): 192-201.

17. Clark E, Hoare C, Tanianis-Hughes J, Carlson GL, and G Warhurst. Interferon γ induces translocation of commensal *Escherichia coli* across gut epithelial cells via a lipid raft-mediated process. Gastroenterology 2005; 128: 1258-1267.

18. Strauman MC, Harper JM, Harrington SM, Boll EJ, and JP Nataro. Enteroaggregative *Escherichia coli* disrupts epithelial cell tight junctions. Infection and Immunity 2010; 78(11): 4958-4964.

19. Bansal T, Alaniz RC, Wood TK, and A Jayaraman. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. Proceedings of the National Academy of Sciences 2010; 107(1).

20. Zhang Q, Li Q, Wang C, Liu X, Li N, and J Li. Enteropathogenic *Escherichia coli* changes distribution of occludin and ZO-1 in tight junction membrane microdomains *in vivo*. Microbial Pathogenesis 2009; 48(2010): 28-34.

21. Simonovic I, Rosenberg J, Koutsouris A, and G Hecht. Enteropathogenic *Escherichia coli* dephosphorylates and dissociates occludin from intestinal epithelial tight junctions. Cellular Microbiology 2000; 2(4): 305-315

22. Shifflett DE, Clayburgh DR, Koutsouris A, Turner JR, and GA Hecht. Enteropathogenic *E. coli* disrupts tight junction barrier function and structure *in vivo*. Laboratory Investigation 2005; 85: 1308-1324.

23. Matsuzawa T, Kuwae A, and A Abe. Enteropathogenic *Escherichia coli* Type III effectors EspG and EspG2 alter epithelial paracellular permeability. Infection and Immunity 2005; 73(10): 6283-6289.

24. Zhang Q, Li Q, Wang C, Li N, and J Li. Redistribution of tight junction proteins during EPEC infection *in vivo*. Inflammation 2012; 35(1).

25. Nusrat A, Parkos CA, Verkade P, Foley CS, Liang W, Innis-Whitehouse W, Eastburn KK, and JL Madara. Tight junctions are membrane microdomains. Journal of Cell Science 2000; 113: 1771-1781.

26. Thanabalasuriat A, Koutsouris A, Hecht G, and S Gruenheid. The bacterial virulence factor NleA's involvement in intestinal tight junction disruption during enteropathogenic *E. coli* infection is independent of its putative PDZ binding domain. Cell Microbiology 2010; 12:31-41.

27. Navarro-Garcia F, Gutierrez-Jimenez J, Garcia-Tovar C, Castro LA, Salazar-Gonzalez H, and V Cordova. Infection and Immunity 2010; 78(10): 4101-4109.

28. Franklin SB and E Latz. For gut's sake: NLRC4 inflammasome distinguish friend from foe. Nature Immunology 2012; 13(5).