

ABSTRACT

Title of Document: ESTROGEN AND PROGESTERONE
ENHANCE NEISSERIA GONORRHOEAE
TRANSMIGRATION ACROSS A
POLARIZED MONOLAYER VIA A
MECHANISM THAT HIJACKS EGFR.

Vonetta Lisa Edwards
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Directed By: Dr. Wenxia Song, Associate Professor
Cell Biology and Molecular Genetics

Gonorrhea, a common sexually transmitted infection, is caused by the gram-negative bacterium *Neisseria gonorrhoeae*. In the female reproductive tract, gonococci (GC) initiate infection at the apical surface of columnar endocervical epithelial cells. These cells provide a physical barrier against mucosal pathogens by forming continuous apical junctional complexes between neighboring cells. This study examines the interaction of GC with polarized epithelial cells. We show that viable, but not gentamicin killed, GC preferentially localize at the apical side of the cell-cell junction in polarized endometrial and colonic epithelial cells, HEC-1-B and T84, respectively. In GC infected epithelial cells, continuous apical junctional complexes are disrupted, and the junction-associated protein β -catenin is redistributed from the apical junction to the cytoplasm and to GC adherent sites. However, GC inoculation does not change the overall cellular level of junctional proteins. This redistribution of junctional proteins is associated with a

decrease in the apical junction's barrier function against the lateral movement between the apical and basolateral membranes, but not against the permeability through the paracellular space. Disruption of the apical junction by removing calcium increases GC transmigration across the epithelial monolayer. GC inoculation induces the phosphorylation of both epidermal growth factor receptor (EGFR) and β -catenin, while inhibition of EGFR kinase significantly reduces both GC-induced β -catenin redistribution and GC transmigration. These results suggest a relationship between junction protein redistribution from the plasma membrane with the resultant weakening of the junctional complex, and an increase in the ability of GC to transmigrate. The presence of the female sex hormones estrogen and progesterone, lead to an increased degree of disruption of the junctional complex and enhance GC transmigration across the monolayer. Therefore, GC are capable of weakening the apical junction and the polarity of epithelial cells via activating EGFR, which facilitates GC transmigration across the epithelium.

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By

Vonetta Lisa Edwards

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Advisory Committee:
Professor Wenxia Song, PhD. Chair
Professor Daniel Stein, PhD.
Professor Ian Mather, PhD.
Professor Xiaoping Zhu, D.V.M, PhD.
Professor Philip DeShong, PhD.

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Dedication

I would like to dedicate my dissertation to my family, my parents (Carol and Alywn Edwards), my sister (Dinessa Edwards) and my grandmother (Muriel Archer). Thank you for teaching me the value of hard work and perseverance and for your unending support and encouragement.

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Table of Contents

Dedication.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
List of Figures.....	vii
List of Abbreviations.....	ix
Chapter 1: Introduction.....	1
1.1 Disease of gonorrhoea.....	1
1.2 Pathogenesis of <i>Neisseria gonorrhoeae</i>	3
1.3 Epithelial cells and the junctional complex.....	8
1.4 EGFR.....	16
1.5 Pathogens and the apical junction.....	20
1.6 Hormone receptors.....	22
1.7 Sex hormones and genital epithelial cells.....	29
1.8 Sex hormones and sexually transmitted infections.....	30
1.9 Rationale.....	33
1.10 Aims.....	34
Aim 1.....	34
Aim 2.....	34
Chapter 2: <i>Neisseria gonorrhoeae</i> Breaches the Apical Junction of Polarized.....	36
Epithelial Cells for Transmigration by Hijacking EGFR	
2.1 Introduction.....	36
2.2 Materials and Methods.....	40
2.2.1 Epithelial Cells.....	40
2.2.2 <i>Neisseria</i> Strains.....	40
2.2.3 Immunofluorescence analysis.....	41
2.2.4 Immunoprecipitation and immunoblotting analyses.....	41
2.2.5 Functional analyses of the apical junction.....	42
2.2.6 GC invasion and transmigration assays.....	43
2.2.7 Statistical analysis.....	43
2.3 Results.....	43
2.3.1 Preferential localization of live gonococci at the cell-cell junction of.....	43
polarized epithelial cells	
2.3.2 GC inoculation disrupts the continuous apical junctional complex.....	44
between polarized epithelial cells	
2.3.3 GC inoculation decreases the fence but not gate function of the apical....	50
junction	
2.3.4 Phosphorylation and redistribution of β -catenin in GC-infected.....	53
epithelial cells	
2.3.5 GC inoculation does not change the cellular levels of junctional proteins	54
2.3.6 Disrupting the apical junction with EGTA increases GC transmigration..	58

across polarized epithelial cells	
2.3.7 GC-induced β -catenin redistribution depends on the kinase activity of EGFR	60
2.3.8 Negative regulation of the apical junction by GC facilitates GC transmigration	63
2.3.9 Invasion of polarized cells is not influenced by EGFR activation	63
2.4 Discussion	65
Chapter 3: The Female Sex Hormones Estrogen and Progesterone Increase <i>Neisseria gonorrhoeae</i> Transmigration across a Polarized Epithelial Monolayer	71
3.1 Introduction	71
3.2 Materials and Methods	74
3.2.1 Epithelial Cells	74
3.2.2 <i>Neisseria</i> strains	75
3.2.3 GC growth	75
3.2.4 Immunofluorescence analysis	75
3.2.5 Functional analyses of the apical junction	76
3.2.6 GC adherence, invasion and transmigration assays	76
3.3 Results	76
3.3.1 Sex Hormones do not affect GC growth	76
3.3.2 Hormones slightly increase the localization of GC clusters at the cell-cell junction	77
3.3.3 Hormones enhance GC-induced redistribution of the junctional protein ZO-1	79
3.3.4 Permeability of the junctional complex is not affected by the presence of hormones	82
3.3.5 Treatment with hormones increases GC transmigration without affecting GC adherence and invasion	82
3.3.6 Inhibition of the classical hormone receptors does not reverse the effect of 17β -estradiol nor progesterone on GC transmigration	86
3.3.7 Inhibition of EGFR in the presence of 17β -estradiol or progesterone affects GC transmigration	88
3.4 Discussion	90
Chapter 4: Conclusions	94
4.1 General Summary	94
4.2 Future Directions	101
Appendices	104
A.1 Delta Opa	104
A.1.1 Lack of Opa affects GC invasion into both non-polarized and polarized epithelial cells	104
A.1.2 GC transmigration was increased in the absence of Opa	106
A.1.3 Predominantly Opa negative bacteria were recovered after transmigration	108
A.2 Layman's Summary	110

Bibliography 111

List of Figures

Fig. 1	Steps in gonococcal infection	6
Fig. 2	Location of the junctional complex	9
Fig. 3	Interaction of junctional proteins and actin with ZO domains	12
Fig. 4	EGFR phosphorylates numerous cytoplasmic proteins	18
Fig. 5	Estrogen and progesterone levels over the course of the menstrual cycle	23
Fig. 6	Estrogen and progesterone hormone receptors	25
Fig. 7	Live GC preferentially localize at the cell-cell junction of polarized epithelial cells	45
Fig. 8	Viable but not killed GC disrupt the continuous apical junction location of ZO-1 and occludin in polarized HEC-1-B cells	46
Fig. 9	GC inoculation disrupts the continuous apical location of ZO-1 and occludin in polarized T84 cells	48
Fig. 10	GC inoculation increases the lateral mobility between the apical and basolateral membrane, but not the permeability of the apical junction in polarized cells	51
Fig. 11	GC inoculation induces the phosphorylation and redistribution of β -catenin from the apical junction to the cytoplasm	55
Fig. 12	GC inoculation does not change the cellular levels of junctional proteins	57
Fig. 13	Disrupting the apical junction by EGTA increases GC transmigration	59

Fig. 14	GC inoculation induces the phosphorylation of EGFR, which is required for GC-induced redistribution of β -catenin	61
Fig. 15	GC transmigration is EGFR dependent	64
Fig. 16	GC invasion is unaffected by EGFR	66
Fig. 17	Sex hormones do not affect GC growth	78
Fig. 18	Hormones slightly increase the localization of live GC at the cell-cell junction, and enhance ZO-1 re-distribution in the presence of GC	80
Fig. 19	Permeability of the junctional complex is not affected by the presence of hormones	83
Fig. 20	Transmigration, but not adherence or invasion is affected by 17β -estradiol or progesterone	85
Fig. 21	Neither 17β -estradiol nor progesterone effects on transmigration are reversed by the presence of classical hormone receptor inhibitors	87
Fig. 22	Inhibition of EGFR kinase activity reduces GC transmigration in the presence of both 17β -estradiol and progesterone	89
Fig. 23	Working model of EGFR activation leading to increased GC transmigration and the proposed synergistic effect of hormones on this process	99
Fig. 24	Lack of Opa affects GC invasion into cells	105
Fig. 25	Increased GC transmigration in the absence of Opa	107
Fig. 26	Opa negative bacteria were recovered after GC transmigration	109

List of Abbreviations

aa	Amino acids
AF	Transactivation domain
AG1478	Tyrphostin AG 1478 (EGFR kinase inhibitor)
AJ	Adherens Junction
BV	Bacterial Vaginosis
Ca ²⁺	Calcium
CFU	Colony Forming Units
CR	Coding Repeats
DBD	DNA Binding Domain
DGI	Disseminated Gonococcal Infection
E-cadherin	Epithelial cadherin
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene glycol tetraacetic acid
ER	Estrogen Receptor
ERK	Extracellular signal related kinase
FBS	Fetal Bovine Serum
FI	Fluorescent Intensity
FIR	Fluorescent Intensity Ratio
FITC	Fluorescein
FRT	Female Reproductive Tract
Gab1	GRB2 associated binding protein-1

GC	Gonococci (<i>Neisseria gonorrhoeae</i>)
GCK	Gonococcal media base with Kellogs supplement
GPCR	G-protein coupled receptor
Grb2	Growth factor receptor bound protein-2
GUK	Guanylate kinase
HB-EGF	Heparin binding – Epidermal growth factor
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HSPG	Heparin Sulfate Proteoglycan
ICI	ICI 182 780 (estrogen receptor antagonist)
JAM	Junction Adhesion Molecules
kDa	kilo Daltons
LOS	Lipooligosaccharide
MAGUK	Membrane-associated guanylate kinase
MAPK	Mitogen Activated Protein Kinase
MCP	Membrane Cofactor Protein
Mif	Mifepristone (progesterone receptor antagonist)
MOI	Multiplicity of Infection
NLS	Nuclear Localization Sequence
NR	Nuclear Receptor
Ω	olms
Opa	Opacity associated protein
PID	Pelvic Inflammatory Disease

PI3K	Phosphoinositide 3 kinase
PIP ₂	Phosphoinositol 4,5 bisphosphate
PIP ₃	Phosphoinositol 3,4,5 triphosphate
PKC	Protein kinase C
Phe	Phenylalanine
PLC	Phospholipase C
PR	Progesterone receptor
PRD	Proline rich domain
RIPA	Radio-Immunoprecipitation Assay
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Ser	Serine
SH	Src Homology
sh RNA	Short hairpin RNA
si RNA	Small interfering RNA
STI	Sexually Transmitted Infection
Thr	Threonine
TEM	Transmission Electron Microscopy
TER	Transepithelial Electrical Resistance
TJ	Tight Junction
TNF	Tumor Necrosis Factor
Tyr/Y	Tyrosine
UTI	Urinary Tract Infection
ZO	Zona Occludin

Chapter 1: Introduction

1.1 Disease of gonorrhea

Sexually transmitted infections (STIs) are a leading cause of diminished healthy living, especially of young women in developing countries. Not only can they lead to serious and sometimes fatal complications, but they also facilitate the increased transmigration of human immunodeficiency virus (HIV) [1, 2]. Gonorrhea is a STI that has been recorded as early as the 18th century. Initially gonorrhea and syphilis were considered the same infection. It was not until 1838 that they were accepted as separate diseases and in 1879 Albert Neisser described the morphological characteristics of the bacteria later identified as the causative agent of gonorrhea [3].

Neisseria gonorrhoeae (GC), a gram-negative bacillus, is an obligate human pathogen. GC generally affects the mucosal epithelia in the urethra in males and the uterine cervix in females. However, it can also infect the rectum, throat (from sexual activity) and eye (from the birth canal) [4]. Complications arising from infection in males are relatively uncommon, as the infection is readily recognized and cleared by the immune system. However, in females approximately 50% of the infections are asymptomatic, and complications can develop from the infections. The most common complication is pelvic inflammatory disease (PID), and approximately 40% of PID cases are associated with a gonococcal infection [5]. Of these 10-15% may lead to infertility as a result of the scarring of the reproductive tract and/or blockage of the fallopian tube [6]. Other syndromes caused by GC include endocervicitis, urethritis, epididymitis, proctitis, pharyngitis and conjunctivitis[1]. This shows that the spectrum of gonococcal infection is

expansive, ranging from a complete lack of symptoms, to apparently insignificant symptoms that can be misdiagnosed as a non-consequential infection to significant and visible symptoms.

While the urethra is the predominant site of all infections in males, in women it is generally the site of symptomatic infections. The female urethra is lined by stratified squamous epithelium with a few areas of columnar epithelium, while the male urethra consists almost exclusively of columnar epithelium, both stratified or pseudo-stratified. GC preferentially infect non-ciliated columnar epithelia, thus making the male urethra an ideal site for primary gonococcal infection [7]. Depending on the location of the infection within the female reproductive tract (FRT), the infection progression can vary. GC infection through the endometrium can lead to a range of manifestations, from an uncomplicated generally asymptomatic infection to a complicated PID situation [8]. The clinical manifestations of gonorrhoea in women include cervicitis (abnormal vaginal discharge), urethritis (painful urination and frequency of urination), acute bartholinitis (labial pain or swelling) or abnormal uterine bleeding and/or lower abdominal pain. Some of these symptoms strongly resemble the clinical presentations of urinary tract infections (UTIs) and as such are ignored or improperly diagnosed [1]. PID has a collection of symptoms, which include, acute endometritis, salpingitis and peritonitis and is caused by dissemination of GC from the cervix to the upper tract of the female reproductive system [3]. The rate of PID in women with a GC infection has increased from 20% in the 1980's [3] to 30-40% in the early 2000s [5]. The dissemination of GC infection beyond the reproductive tract can lead to disseminated gonococcal infection (DGI), which can manifest as skin lesions, asymmetrical arthralgias, tenosynovitis or arthritis [9].

Antibiotics are the most effective treatment for gonorrhoea. One of the earliest treatments was prontosil, a sulfonamide that was introduced in 1935. Penicillin was used when over 25% of patients no longer responded to prontosil. However, by 1955 there were reported cases of gonococcal penicillin resistance [10, 11]. After these reports *tetracycline*, *chloramphenicol*, *streptomycin* and *spectinomycin* were all tried [3]. The current recommended treatments include cefixime, ceftriaxone or cephalosporin. There was an increase in GC resistance to antibiotic treatment observed in 2010 to all antibiotics, suggesting the possibility of a developing superbug [6] and by extension a health crisis, since the current line of treatment is the last line of defense available. Due to a high level of coinfection with Chlamydia, gonococcal treatments sometimes include regimens that are also effective against uncomplicated chlamydia e.g. azithromycin or doxycycline [6, 12].

1.2 Pathogenesis of *Neisseria gonorrhoeae*

GC pathogenesis has been extensively studied in non-polarized epithelial cell culture *in vitro*, and a general consensus has been attained on the basics of the process. Within the first hours of infection GC appear to preferentially attach to the microvilli of non-ciliated columnar epithelial cells, where the microvilli are observed not only in contact with the GC, but also surrounding them. This initial attachment appears to be mediated by the GC type IV pili [13]. Pili are filamentous polymers that are approximately 6 nm in diameter and can be several microns in length. These fibers are not only involved in attachment, but also in GC aggregation and twitching motility, among other processes [8, 14]. CD46 (also called membrane cofactor protein-MCP) was

the first recognized receptor of pili and is found on epithelial, endothelial and sperm cells [4, 15]. Based on its significant role in GC adherence to host cells, variations of pili factors have been shown to have a significant effect on GC pathogenicity [4].

After initial attachment, host cell lamellipodia and ruffles are observed at the site of GC adherence [8]. GC then become intimately attached to the host membrane [4, 16, 17]. Opacity protein (Opa, or protein II) plays a role in strengthening the interactions between the GC and the host cells as well as interactions within the gonococcal microcolonies [3]. Due to the numerous variants of Opa that are present within a given microcolony, the effect of one specific Opa variant is difficult to elucidate, even though it is known that different expressed Opas have differing effects on GC-host interactions and overall pathogenesis [18]. Opa proteins can bind to numerous receptors on the host cells, including heparan sulfate proteoglycans (HSPGs) that facilitate the recruitment of F actin and tyrosine phosphorylated proteins to the site of attachment [19], and CD66 that can lead to GC uptake and activation of signaling cascades [20-22].

Lipooligosaccharide (LOS) is another GC surface component involved in pathogenesis. GC interacts with host surface receptors such as asialoglycoprotein receptor [23-25] and triggering receptor expressed on myeloid cells-2 (TREM-2) [26] via LOS. The presence of LOS also allows GC strains lacking Opa to still invade into host cells and this invasion is enhanced by the presence of the lacto-*N*-neotetrose [27].

GC are then engulfed by the host cells [4], where they are observed to be both cytoplasmic and within vacuoles [17, 28, 29]. Though piliated GC adhere better to host cells, there is no observable difference between the efficiency of internalization of piliated compared to non-piliated GC, once GC attachment has been established [28]. It

was observed by Timmermann *et al.* that after inoculation for the same amount of time, approximately 20 fold more GC adhered to endometrial cells than invaded them [8]. The mechanism of internalization appears to require the actin cytoskeleton, since treatment with the actin polymerization inhibitor cytochalasin D inhibited GC internalization. It was observed by Shaw *et al* that while *N gonorrhoeae* MS11 and F62 (pathogenic strains) readily invaded into host epithelial cells, *N lactamica* (commensal strain) did not significantly invade even after 12 h of incubation [28].

Once internalized from the apical (top) surface of the cell, the bacteria multiply within and traverse to the basal (bottom) membrane. In non-polarized cells and organ culture, transcytosis across the monolayer occur over a 24-48 h period [16, 17, 28]. Vacuoles containing GC are seen to fuse with the basal membrane and the bacteria are released into the underlying layer. These bacteria can then attach to and invade these deeper epithelial layers [17]. In addition to its role in invasion, LOS also binds to and activates dendritic cells via the SIGN (CD209) receptor [30]. GC variation of its LOS sequence can lead to changes in cytokine production [31] and be used as a mechanism to evade the host immune response [30, 32]. This extensively studied pathogenicity pathway of GC in non-polarized cells is shown in Fig. 1.

Analysis of GC pathogenesis in polarized cells has not been as extensively analyzed. However, from the work that has been performed transmigration of GC across a polarized monolayer was shown to occur as early as 10 h post inoculation [33], with a mid-range of 24 h [34] and as late as 36-48 h [35]. A comparison of transmigration (total number of bacteria that cross from the apical to basal side), versus traversal (bacteria that invade and then exocytose), showed a two-fold higher recovered CFU from

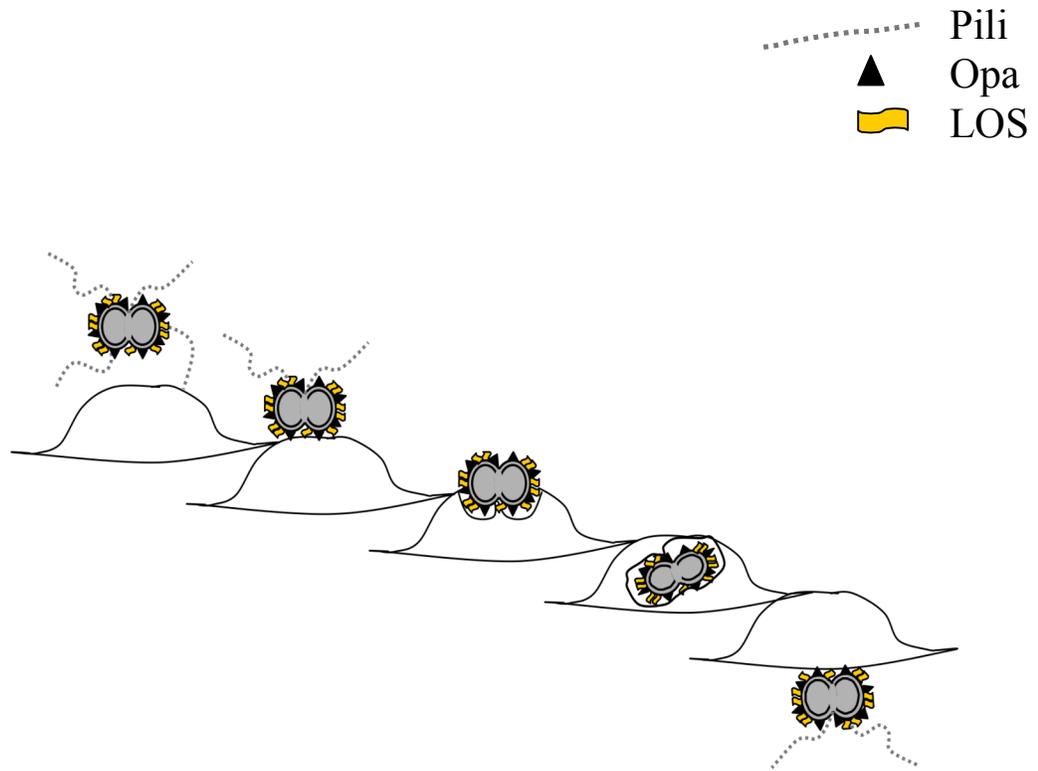


Figure 1. Steps in gonococcal infection. This model shows the epithelial cells apically inoculated with GC. Bacteria initially attach using pili, intimate attachment occurs via Opa and/or LOS interaction with host surface receptors. Bacteria are then internalized and can transcytose and be released from the basal surface.

transmigration assays [34]. This suggests that GC might cross the monolayer by mechanisms additional to invasion/exocytosis. Both pili [35] and the presence of *fit* genes [33] appear to enhance GC transmigration. Similar to invasion, the actin cytoskeleton is also involved in GC transmigration as actin inhibitors reduce the ability of GC to transmigrate [34]. The integrity of the monolayer, as observed by transepithelial readings and permeability to dyes, was not affected by the passage of GC [34, 35]. Due to experimental difficulties and differences between the numerous GC strains and variants used, a consensus has not been attained with regards to the mechanism(s) used by GC for transmigration.

GC is a phase and antigenic variable organism, with the majority of its surface proteins capable of varying at a high frequency. The definition of phase variation is that the gene expression is controlled by an on versus off switch, while antigenic variation means that the primary sequence of a protein is being changed. Phase and antigenic variation is observed in many bacteria and even in pathogenic viruses and parasites. This constant changing of the surface molecules confers on GC a survival advantage by facilitating evasion of the immune system and must be considered a significant feature of GC infection [4]. Gonococcal surface molecules, Pili (P^+), Opa (O^+) and LOS, have been shown to play essential roles in pathogenesis, and phase vary at $\sim 10^4$ [36], $\sim 10^3$ [37] and $\sim 10^4$ [38] respectively. The variability of the surface molecules also affects GC-GC interactions, consequently changing the morphology of the bacterial microcolonies. Based on colony morphology and surface expression, Kelloggs originally designated GC as T1 (P^+O^-), T2 (P^+O^+), T3 (P^-O^+) and T4 (P^-O^-). These classifications were established using the cultured exudate from a male patient [39].

Variation of Opa occurs due to the presence of a repetitive coding repeat (CR) sequence. The number of repeats that are present determines whether the reading frame for a specific Opa is in or out of frame and therefore whether the protein is translated [37]. CR sequences are independent for each Opa gene and thus autonomous of each other [40]. Pilin variation is due to the homologous recombination event between several silent gene copies (*pilS*) and the expressed pilin gene (*pilE*) [41, 42]. Production of truncated S-pilin, which is secreted instead of polymerized into the pilin, can occur [43]. LOS also undergoes variation. There are poly-G tracts found within the coding frames responsible for glycosyl transferase. Slippage of these tracts can cause termination of transferase translation and thus LOS structure [44].

1.3 Epithelial cells and the junctional complex

Initially it was believed that the barrier formed between polarized cells was simply a thickening of the intracellular areas involved in the contact, and this unregulated seal was termed the ‘terminal bar’. Using electron microscopy (EM), it was found that there are actually several independent and distinct junctions that function together, and these were later named the tight junction (TJ), adherens junction (AJ), desmosome and gap junction [45, 46]. The most apically localized of these junctions is the TJ. The TJ and the AJ together form the apical junction (Fig. 2). The apical junction has two functions: to create a diffusion barrier to small solutes and to separate and maintain proteins and lipids in their correct apical or basolateral location in the cell membrane [45, 47, 48]. Different imaging techniques illustrate different characteristics of the TJ. Under transmission electron microscopy (TEM), TJs appear as ‘spot like’ contacts, while freeze

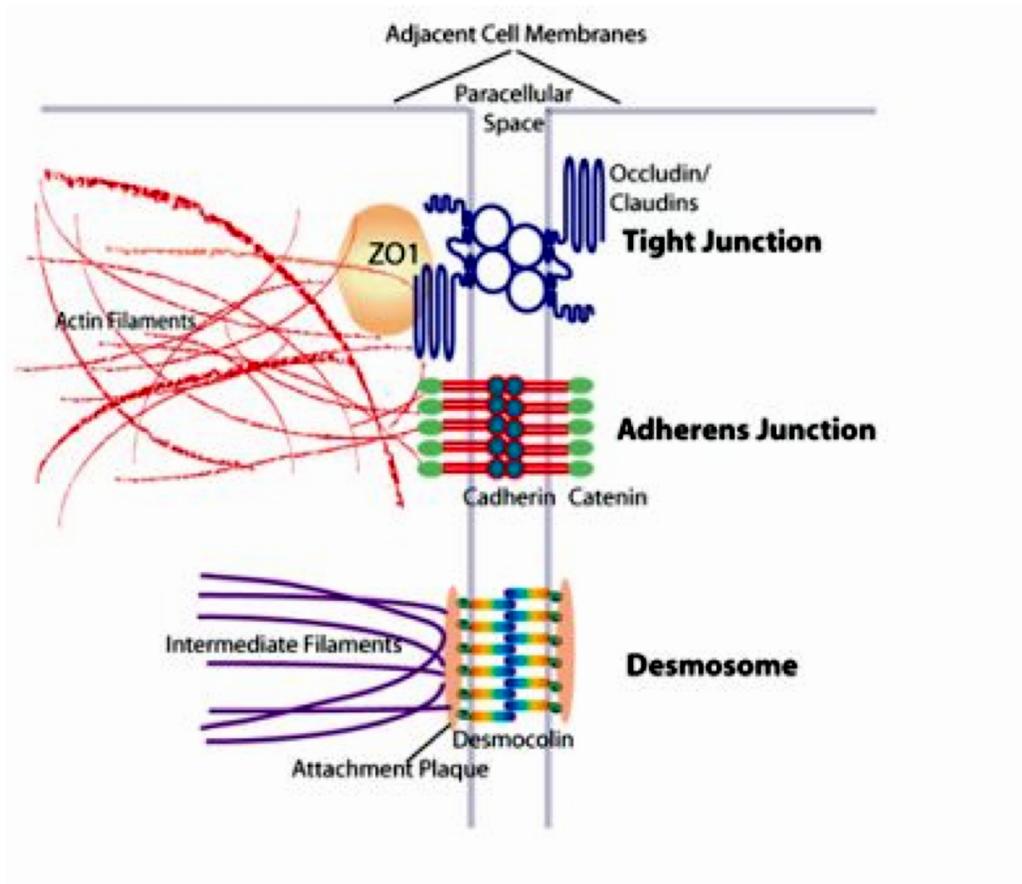


Figure 2. Location of the apical junctional complex. This modified figure shows the location and proteins that comprise the Tight junction (TJ) and Adherens junction (AJ). Modified from http://www.trinity.edu/departament/research/images/mking_graphics.jpg.

fracture electron microscopy show rows of molecular particles that form a continuous branching network of parallel and interconnected strands, localized at the contacts between apposing cells [45, 46, 49]. It has been theorized that the density of these networks directly affects the functionality of the TJ, since the denser the network, the greater the transepithelial electrical resistance (TER) and the less paracellular solute permeability observed [50]. The strength of the TJ barrier varies depending on the types of epithelium and generally relates to the physiological function of the epithelium. If there is a low level of permeability and a high TER, then the junction is considered 'tight', while if the permeability is high and TER is low, the junction is considered 'leaky' [45, 47]. Electron microscopy analysis of the AJ shows parallel apposing membranes with an intercellular space that consists of numerous cylinder-like appendages. Additionally, the cytoplasmic region of the AJ consists of a plaque of proteins and actin filaments [51].

The TJ consists of transmembrane and cytoplasmic proteins. The transmembrane proteins, including occludin, claudin and junction adhesion molecules (JAMs) are assembled together and associate with the actin cytoskeleton via the cytoplasmic zona occludin (ZO) proteins [46, 52]. It has been demonstrated that occludin is involved in cell-cell adhesion and the permeability barrier. Even though occludin appears to be a consistent component of all TJs, sequence comparison across various species shows a surprisingly high divergence in the amino sequence [45]. This 65 kDa protein has four transmembrane domains, two extracellular loops involved in TJ function and cell-cell adhesion and the cytoplasmic amino (N) and carboxy (C) tails. Extracellular loops of occludin on apposing cells interact with each other in a zipper-like fashion, creating the

paracellular seal [45, 46, 53]. McCarty *et al* and Balda *et al* demonstrated the function of occludin by transfecting occludin into cells. This led to an increase in the number of occludin fibers and TER of the cells [53, 54]. There are multiple isoforms of occludin, providing an explanation for the diversity of paracellular permeability among various types of epithelia [52]. While the C terminus is not required for the barrier function, the last ~150 amino acids of the C terminus are required for occludin's localization at the TJ [46]. Phosphorylation of occludin regulates its functionality. Occludin in polarized cells is phosphorylated at its serine (Ser) and threonine (Thr) residues with much lower levels of tyrosine (Tyr) phosphorylation. Upon disruption of the junctional complex, there is a decrease in Ser/Thr phosphorylation, but an increase in phosphorylation of the Y398 and Y402 residues in the C terminal domain [53].

ZO-1 is the most studied TJ protein. This 220 kDa phosphorylated protein is found in all cells, while the 160 kDa ZO-2 is found only in cells that have formed tight junctions. These two proteins are members of the membrane-associated guanylate kinase (MAGUK) protein family. The MAGUK family consists of proteins that are associated with the plasma membrane at regions involved in cell-cell contact, and likely provide scaffolds for organizing TJ transmembrane complexes. This most likely results due to the presence of multiple protein-protein interaction domains [45, 55]. ZOs have multiple PDZ domains that interact with the hydrophobic motifs of their target proteins. Each also contains a Src-homology (SH) 3 domain that mediates protein-protein interactions by binding to proline rich domains (PRD). The ZOs also have a region that shares homology with the guanylate kinase (GUK) enzyme [45, 46]. Mutagenesis analyses indicate that

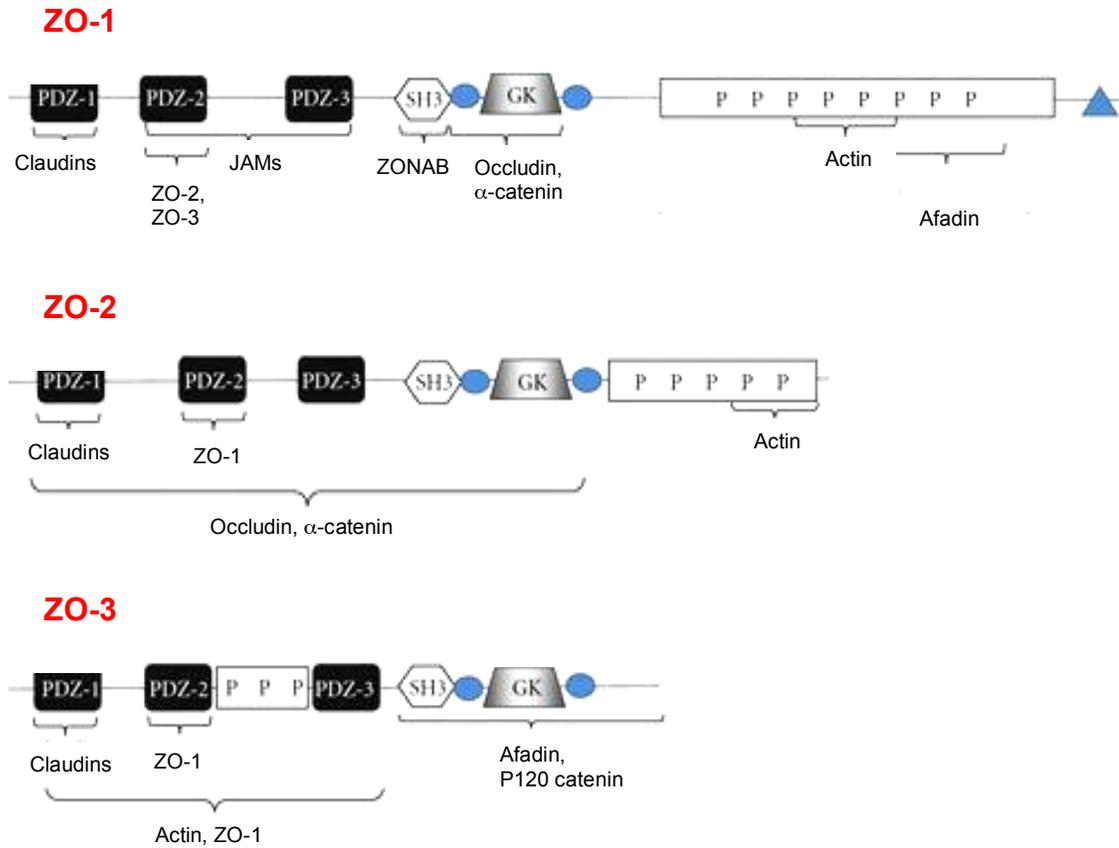


Figure 3. Interaction of junctional proteins and actin with ZO domains. ZO proteins function as scaffolding proteins, linking the junctional complex proteins to the actin cytoskeleton. Modified from Bauer *et al* (2010). Jour of Biomedicine and Biotechnology: 402593.

ZOs are also involved in organizing and facilitating signal transduction. ZO-1 binds occludin via its GUK region, while binding claudin and JAM via the PDZ domain (Fig. 3). Essentially ZO binds these proteins via its N terminus and the actin cytoskeleton via its C terminus. This links the TJ to the actin cytoskeleton and facilitates the assembly of the junction. Junction-associated actin and its motor myosin, which form a supporting ring around the apical junction, can exert a contraction force on TJs, causing junctional proteins on apposing cells to move away from each other. This can lead to weakening of the junctional complex and increase permeability via the paracellular space [46].

The adherens junction (AJ) is localized immediately beneath the TJ. Differing from TJs there is a visible space between the membranes of opposing cells when observed under an electron microscope. Similar to the TJ, the AJ links the membrane and cytoskeleton at sites of cell-cell contact [46, 51]. The formation of the AJ has been shown to be required for the assembly of the TJ, and the stability of the AJ is important for the continued maintenance and functionality of the TJ. The major components of the AJ are members of the cadherin super family. This family of proteins can be divided into six sub families: classical cadherins type I (E, N, P, R-cadherins); classical cadherins type II (cadherin-6 to 12); desmosome cadherins (desmocollins, desmogleins); cadherins with a very short or no cytoplasmic tail; protocadherin and distinctly related products [56]. In epithelial cells the major functional cadherin member is a classical Type I, E-cadherin. It is a 120 kDa, single pass transmembrane glycoprotein that mediates intercellular adhesion in a Ca^{2+} dependent manner [46, 51]. E-cadherin has 5 ectodomains (EC1-5) that bind to Ca^{2+} and mediate trans-homophilic interactions between E-cadherin on neighboring cells. Its 150 aa cytoplasmic tail is highly conserved and binds to the actin

cytoskeleton and cytoplasmic signaling molecules including catenins and α -actinin. E-cadherin binds to the armadillo sequence repeats of β -catenin, and this interaction is required for the transport of newly synthesized E-cadherin to the plasma membrane [46].

β -catenin is a member of the catenin family which consists of alpha, beta and gamma catenin. It is a 90 kDa protein that consists of 13 repeats of a 42 aa armadillo sequence. It also has a highly conserved serine (Ser) rich region. The phosphorylation of three conserved serines increases its affinity for E-cadherin. In contrast, the phosphorylation of tyrosine at positions 489 and 654 disrupts its interaction with E-cadherin [51, 56, 57]. It has been shown that the Tyr phosphorylation of β -catenin by pp60^{c-src} led to a 5-fold decrease in its ability to bind to E-cadherin and mutation of Tyr654 to phenylalanine (Phe) negated this decrease [58]. This makes β -catenin a central component of the AJ architecture [56]. The final armadillo repeat forms a long helix with a positively charged groove that accommodates the cytoplasmic tail of E-cadherin and binds via ionic interactions [58]. The interaction between E-cadherin and β -catenin appears to be the rate-limiting step to the establishment of the junctional complex [46]. Additionally, the interaction between E-cadherin and β -catenin has a protective effect against the proteolysis of E-cadherin [51].

In epithelial cells, E-cadherin, β -catenin, α -catenin and ZO-1 first assemble at the junctional complex, followed by recruitment of occludin and claudin [59]. Disassembly of both the TJ and AJ is caused by directly modifying components of both junctions, or by indirect effects mediated via the cytoskeleton. Detectable signs of the disassembly of the junctional complex include decreased TER, increased permeability of molecules, displacement of ZO-1 and occludin from the TJ and redistribution of E-cadherin and β -

catenin from the AJ [60-62]. Disassembly can occur by three main methods. Firstly there is signaling that leads to movement of junctional proteins away from the junctional complex. Increased phosphorylation of both ZO-1 and β -catenin is responsible for the redistribution of these proteins away from the junctional complex. The phosphorylation of β -catenin prevents it from binding to E-cadherin, and this essentially disrupts the foundation of the junctional complex. Additionally, disruption of the E-cadherin- β -catenin interaction disconnects the link between the AJ and the actin cytoskeleton, thereby further affecting the overall stability of the junctional complex [46].

Phosphorylation of β -catenin also activates its transcriptional activity and appears to cause downregulation of ZO-1 [63]. However, since the half-life of these proteins range from 6-12 h, regulation at the transcription level cannot account for the observed rapid disassembly of the junction [46].

Secondly there is endocytosis of the junctional proteins. Intracellular accumulation of both ZO-1 [64, 65] and cadherin [66, 67] has been reported, providing evidence for this mechanism. Additionally, proteins known to be involved in endocytosis, such as Rab13, VAP33 and Sec6/8 have been shown to regulate the assembly of the junctional complex [68-70]. Various pathogens, either directly or indirectly cause increased internalization of both TJ and AJ proteins [71]. There can be constitutive low-level internalization of proteins such as E-cadherin and occludin, which are continuously shuttling between the junctional complex and endosomes [72-74]. Selective internalization of TJ proteins as seen in intestinal epithelial cells exposed to *E.coli* cytotoxic necrotizing factor-1 or IFN γ [75] can also occur. Previous studies have shown that all major endocytosis mechanisms; clathrin mediated, caveolin and

macropinocytosis, are involved in junctional complex internalization. It has been proposed that different endocytosis pathways internalize junctional proteins at different rates, target them to different subcellular compartments and thus cause different levels of junctional disruption [71].

Finally, there can be complete internalization of both TJ and AJ proteins as seen when cells are depleted of Ca^{2+} [76, 77]. A chemical means of disrupting the junctional complex is provided by the addition of the Ca^{2+} chelator, EGTA. In the absence of EGTA, the TJ proteins (ZO-1, occludin, claudin) appeared exclusively at the membrane as a continuous network, while a small amount of AJ proteins (E-cadherin, β -catenin) were localized in the cytoplasm. After EGTA treatment there was diffuse localization of ZO and occludin at the junction, while E-cadherin and β -catenin were observed throughout the cell. Removal of EGTA led to complete and rapid reassembly of the junction proteins [78].

1.4 EGFR

The function of the TJ and AJ can be regulated by external or internal signals transmitted through cellular signaling pathways. A major manipulation is phosphorylation of junctional proteins. In epithelial cells, ErbB receptors are key surface receptors that control cell survival and replication. A link between activation of the ErbB family of receptors and junction disruption has been established in carcinomas [79].

The ErbB family consists of four members, EGFR/ErbB1/HER1; ErbB2/Neu/HER2; ErbB3/HER3; ErbB4/HER4. They contain an extracellular domain responsible for ligand binding and dimerization, a transmembrane domain and a

cytoplasmic domain that contains a tyrosine kinase domain and multiple phosphorylation sites. Ligand binding induces the formation of receptor homo and heterodimers, which leads to the activation of the tyrosine kinases and trans-autophosphorylation of tyrosines in the cytoplasmic tail. ErbB2 does not bind to ligand and cannot form homodimers, but it is capable of forming heterodimers with other family members. ErbB3 is only functional when it forms a heterodimer [79]. Downstream signaling molecules bind to the phosphorylated tyrosines in the cytoplasmic tail of the receptors (Fig. 4). Many signaling molecules bind to these receptors and each binds to specific sites on particular family members. This in turn initiates specific and conserved signaling cascades [80].

Ligands that bind to the ErbB family of receptors are loosely grouped into three (3) groups: First, those that bind specifically to ErbB1/EGFR including epithelial growth factor (EGF) and tumor necrosis factor alpha (TNF α), second, those that bind to both ErbB1 and ErbB4 including heparin binding EGF (HB-EGF) and epiregulin (EPR) and finally neuregulins (NRGs) with NRG1 and 2 binding to ErbB3 and B4, and NRG 3 and 4 binding to ErbB4 [79].

ErbB1/EGFR is the most studied member of this family of receptors. EGFR can bind to its ligands with two different affinities, however, low-affinity binding constitutes approximately 90% of all binding [81]. There are six tyrosine residues that can be auto-phosphorylated and four that can be phosphorylated by src kinases. The specific residue that is phosphorylated depends on the ligand present, ligand concentration and dimerization partner. The major cytoplasmic proteins that bind to phosphorylated EGFR are PLC γ , Grb2, Gab1 and Shc. The cytoplasmic protein that binds to EGFR determines

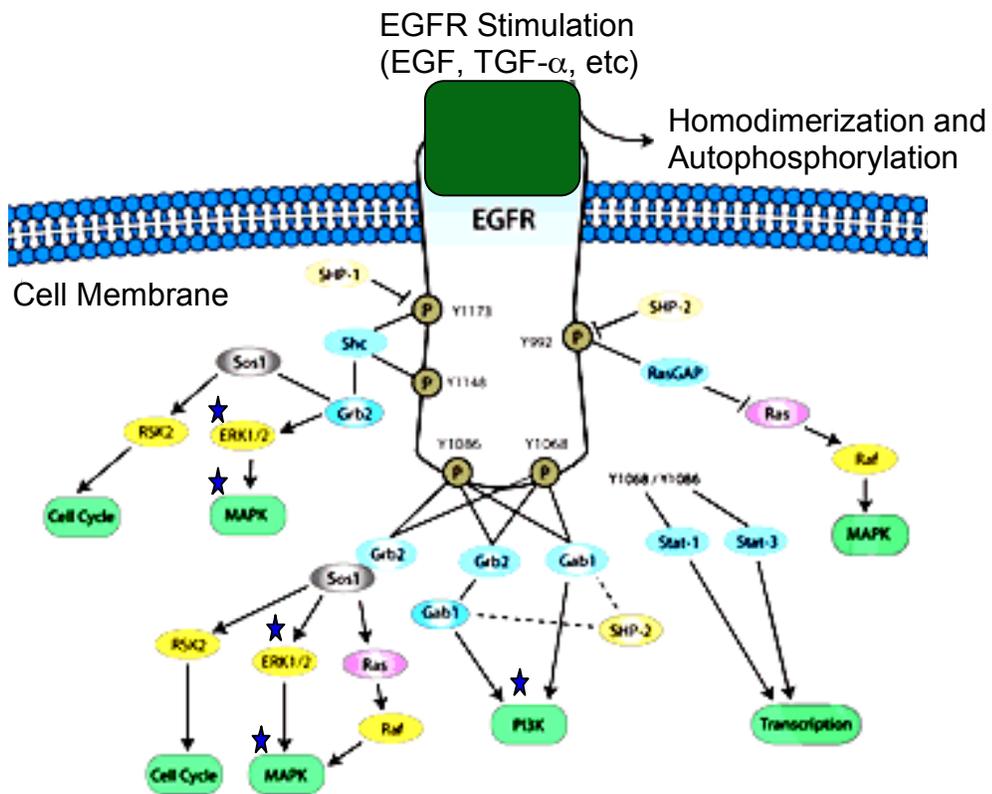


Figure 4. EGFR phosphorylates numerous cytoplasmic proteins. When the EGFR receptor dimerizes its kinase activity is activated and many cytoplasmic proteins are tyrosine phosphorylated. The cytoplasmic proteins interact with tyrosine molecules at specific positions so different pathways are activated based on which tyrosine molecule is involved. Modified from http://www.biomol.de/wiki/index.php=EGFR_Pathway_Map.

the signaling pathway that is activated. Binding of Grb2 and/or Grb2/Shc leads to activation of the MAPK and phosphoinositide 3 kinase (PI3K) pathways. Binding of PLC γ leads to cleavage of phosphoinositide 4,5 biphosphate (PIP₂) and the resultant activation of protein kinase C (PKC) [82]. Interaction with Gab1 induces activation of phosphoinositide 3,4,5 triphosphate (PIP₃) and the downstream activation of the AKT pathway [83]. These cascades lead to numerous outcomes including cell proliferation and differentiation, cell metastasis, apoptosis suppression and calcium modulation [80]. Different binding affinities also lead to activation of different signaling cascades, with low-affinity binding apparently being the preferential binding for activation of intracellular signaling including Ras/MAPK and PI3K/Akt signaling pathways [84].

EGFR is predominantly expressed at the basolateral surface of polarized epithelial cells [85], but functional EGFR receptors have been detected on the apical surface of epithelial monolayers [86]. Proteins such as Erk show no difference in Tyr phosphorylation when basally or apically located EGF receptors are activated [86, 87]. Ligand binding also induces EGFR internalization as a signaling down-regulation mechanism [88]. It appears that internalization of EGFR is more efficient on the basolateral side, which coincides with the observation that there is more efficient down-regulation of basolateral EGFR [87]. Basal exposure of EGFR ligands causes transient signaling, while apical exposure leads to sustained signaling [86], even though receptors at the basolateral and apical surfaces have similar abilities to activate signaling [87]. EGFR activation induces the Tyr phosphorylation of β -catenin. Yasmeen *et al.* demonstrated that the phosphorylation of β -catenin leads to dissociation of the E-cadherin- β -catenin complex from the actin cytoskeleton, thus disassembling the AJ [79].

Takahashi *et al.* showed that both E-cadherin and β -catenin were accumulated at the cell-cell junction and interacted with each other in confluent monolayers of human breast epithelial cells (HBE). Tyrosine phosphorylation of β -catenin was reduced when non-confluent cells became confluent, however overall levels of the proteins were essentially the same. This suggests that for confluence and proper monolayer formation β -catenin must be unphosphorylated [89]. It was also shown that β -catenin was Tyr phosphorylated to a greater level when cells were apically exposed to EGF as compared to basolateral exposure [87].

Previous research in our lab has shown that EGFR transactivation, induced by the presence of either pili or opa, is required for invasion of GC into non-polarized cells. GC induces increased shedding of EGFR ligands and leads to activation of the downstream protein Erk [90]. Activation of EGFR leads to disruption of the junctional complex via phosphorylation of the junctional protein β -catenin [79] and in polarized cells the usually basolaterally localized EGFR is recruited to GC on the apical surface [90].

1.5 Pathogens and the apical junction

The epithelium is a critical barrier that prevents pathogenic entry into the human body. Pathogens have evolved means to directly or indirectly breach this barrier by affecting the apical junction. Interference with cell-cell junctions destabilizes the structure and function of the epithelium and facilitates the paracellular passage of the pathogen across the protective epithelium into deeper tissues. There are generally four (4) main mechanisms of interference – 1) Secretion of enzymes that can modify the

extracellular portion of the junctional proteins. *Vibrio cholerae* secretes the metalloprotease hemagglutinin/protease (HA/P) that cleaves the extracellular domain of occludin [46]. *Bacteroides fragilis* secretes a metalloprotease that cleaves the extracellular domain of E-cadherin and induces the proteolysis of the intracellular domain [91]. 2) Secretion of toxins that enter epithelial cells by endocytosis and alter intracellular signaling. *Clostridium difficile* produces toxins A and B that inactivate RhoA, causing actin reorganization and dissociation from the junctional complex. ZO-1 and occludin then dissociate from the TJ and cause a decrease in TER [46, 91]. *Helicobacter pylori* uses its type IV secretory system to inject the effector CagA into epithelial cells where it becomes phosphorylated and gains the ability to interact with ZO-1 at the junctional complex. Additionally CagA induces the internalization and redistribution of E-cadherin, which disrupts the AJ [91]. 3) Injection of proteins that directly destabilize the junctional complex. *Vibrio cholerae* produces the zonula occludin toxin (Zot), which increases the permeability of the TJ [46]. *Clostridium perfringens* produces the toxin CPE that binds to claudin leading to its degradation [91]. *Listeria monocytogenes* expresses the surface protein InA and InB. InA interacts with E-cadherin and induces the redistribution of α and β -catenin away from the AJ. InB disrupts the apical junction by inducing actin reorganization via activating PI3K [91]. 4) Pathogen invasion and direct interaction with the junctional complex. *Escherichia coli* has been shown to cause TER decreases and ZO-1 and occludin dissociation from TJ [46, 91]. *Toxoplasma gondii* accumulate around the junctional complex as an essential part of pathogenesis and are able to cross the epithelial barrier. It has been suggested that it is able to disrupt the junctional barrier without destroying the junctional integrity [91].

The interaction of GC with polarized epithelial cells has not been well studied, so whether GC are capable of interfering with the apical junction and disrupting the epithelial barrier is not known. Early organ culture studies of the male urethra have shown GC in the subepithelial connective tissue. Penetration into underlying tissues can occur via the intercellular spaces, as these spaces appear to be larger in the areas directly affected by GC [7]. No studies in polarized cells have exclusively examined the effect of GC on the junctional complex. The ‘gate’ function of the junction has been peripherally analyzed in conjunction with transmigration analysis, but a detailed look at GCs effect on junctional proteins has not been undertaken. Transmigration of the other pathogenic *Neisseria* species, *Neisseria meningitidis*, shows direct effects on the junctional proteins. Endothelial cells show recruitment of ZO-1, ZO-2 and claudin (TJ proteins) and VE-cadherin, p-120 catenin and β -catenin (AJ proteins) away from the junction and targeted to sites under the bacterial microcolonies [92]. Occludin was cleaved and disassociated from the membrane into the cytoplasm [93]. These findings suggest that GC may have a similar effect on epithelial junctional proteins and my project elucidated these effects.

1.6 Hormone Receptors

The barrier function of the epithelial cells in the female reproductive tract (FRT) is regulated by the hormonal cycle via hormone receptors. Over the course of the menstrual cycle the female sex hormones estrogen and progesterone fluctuate (Fig. 5). The menstrual cycle begins on the first day of vaginal bleeding. Both hormones are at low levels on this day. Approximately on day 5 estrogen levels begin to rise and peak

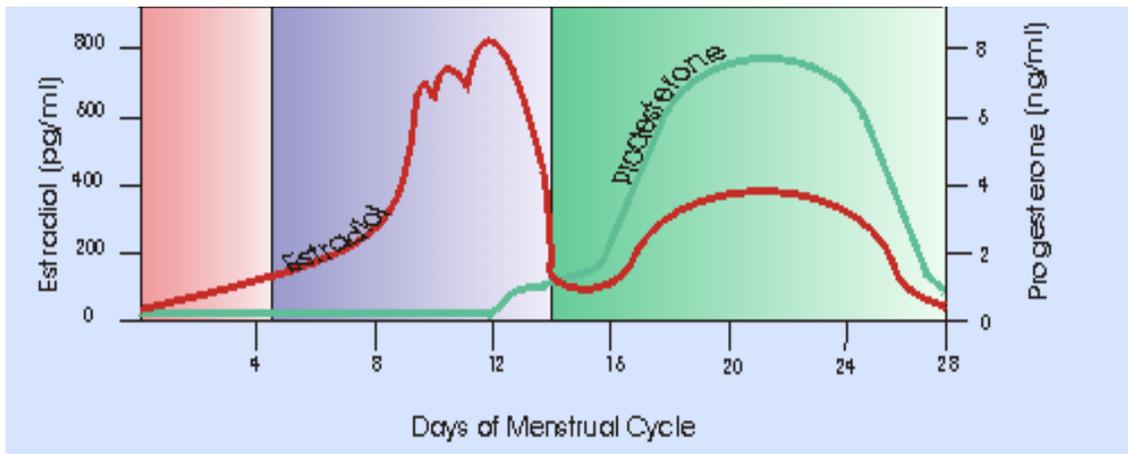


Figure 5. Estrogen and progesterone levels over the course of a menstrual cycle.

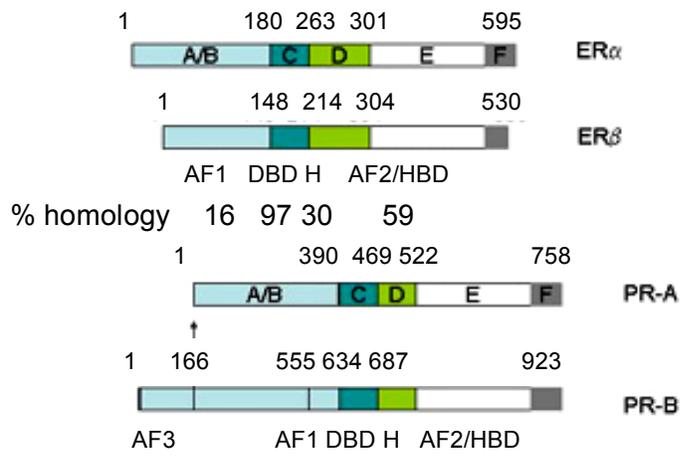
The levels of both estrogen and progesterone vary as the menstrual cycle progresses peaking at two independent stages of the cycle. Both the peaks and the approximate serum hormone levels are indicated. Modified from <http://www.women-health-info.com/231-Endocrinology-Menstrual-Cycle.html>

at ~ 0.2 ng/ml on day 13. Levels steeply decline and begin to rise again, peaking at the lower level of 0.1 ng/ml on days 20-22. Progesterone levels remain low until day 14 when they slowly begin to rise, peaking on day 20-22 at ~9 ng/ml. If conception does not occur both estrogen and progesterone levels rapidly decline and the cycle restarts [94-96]. The presence of estrogen and progesterone has been shown to affect the actin cytoskeleton [97, 98] and to increase occludin cleavage [99] thus decreasing the barrier function of the genital cells. During the later stages of the cycle when progesterone is at an elevated level the endometrial epithelium thickens, however the intercellular spaces are also increased [94].

Estrogen (ER) and progesterone (PR) receptors are two of the major hormonal receptors. They are part of the nuclear receptor (NR) superfamily, specifically Class I NRs [100]. There are three major functional regions: a N-terminal transactivation domain (AF1) that functions independent of ligands, a central DNA binding domain (DBD) that is also responsible for receptor dimerization, and a C-terminal hormone binding domain (HBD) that also serves as an interaction site for co-activators and co-repressors. Another transactivation domain (AF2) and a hinge region connect the HBD and DBD (Fig. 6) [100, 101]. There are two isoforms of both the ER and PR. The ER subtypes (ER α and ER β) are from two different genes, where as the PR isoforms (PR-A and PR-B) arise from a single gene that has two different promoters leading to the production of two separate mRNAs [102].

The DBD and HBD of the two ER subtypes share high homology but the N-terminus with its multiple Ser/Thr phosphorylation sites and AF1 domain, has little homology between the two subtypes. The C-terminus prevents receptor dimerization and

A



B

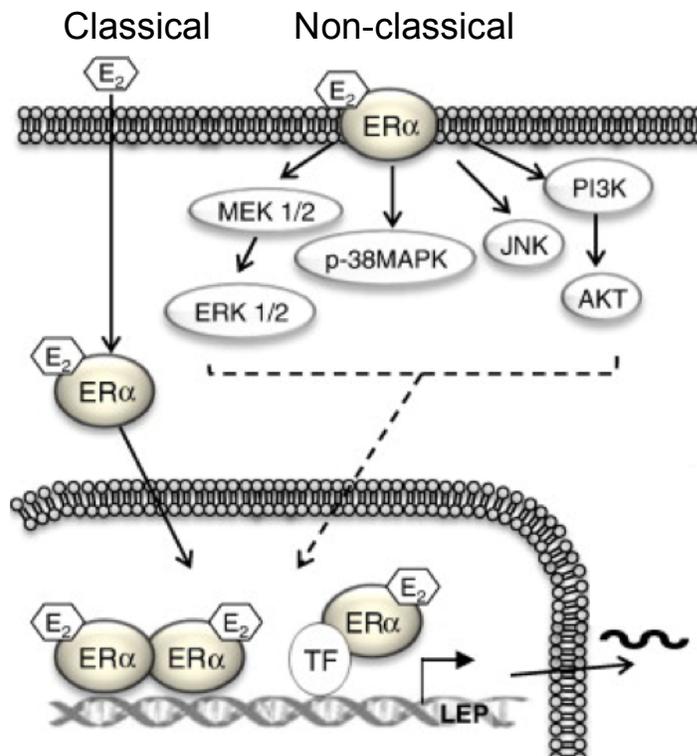


Figure 6. Estrogen and progesterone hormone receptors. (A) ER and PR have high a homology to each other at the DBD, with significantly reduced homology at the other domains. (B) Activated ER can signal through the ‘classical’ pathway that leads to transcription or the ‘non-classical’ pathway that can lead to rapid events due to activation of numerous signaling cascades. AF – Trans-activation domain, DBD – DNA binding domain, H – Hinge region, HBD – Hormone binding domain. Modified http://www.bcm.edu/cms_web/332//rp1.jpg and <http://ars.els-cdn.com/content/image/1-s2.0-S0167488912000237-gr9.jpg>.

thus improper ligand activation. While they recognize similar DNA sequences and respond in a similar manner to the presence of 17β -estradiol, they are functionally distinct and expressed at levels in different tissues [100, 102]. $ER\alpha$ is a more potent transcription activator than $ER\beta$ and $ER\beta$ is capable of reducing transcriptional activity of $ER\alpha$ [102]. 17β -estradiol is the main ER ligand and plays a significant role in sexual development, behavior, reproductive functions, proliferation and differentiation. It binds with high affinity to ER, while its metabolic products such as estrone and estriol bind with a much lower affinity. The effect of 17β -estradiol can be pharmacologically attenuated by the use of anti-estrogens, ER antagonists i.e. ICI 182780 and selective estrogen receptor modulators (SERMs) i.e. tamoxifen [100, 103].

PR-A and PR-B have almost identical DBD and HBD, however PR-A has a truncated N-terminus that is missing 164 aa. PR-B is a strong transcription activator while PR-A, in a manner similar to $ER\beta$, can attenuate the activity of not only PR-B, but also other members of the NR family including the ER. The truncated segment of PR-A contains a third transcription activation domain (AF3), which is responsible for the increased activity observed in PR-B [100, 102]. Similar to the ERs, both PR-A and PR-B are co-expressed in most cells but the ratio depends on the cell type and conditions [102]. The physiological ligand of PRs is progesterone and it plays a major role in differentiation of the endometrium, in preparation of the uterus for implantation and maturation of mammary epithelium. Mifepristone (RU486) is a pharmacological PR antagonist that is commonly used as a contraceptive and in the treatment of benign uterine tumors [100, 104].

Traditional or 'classical' signaling through the NRs results from the binding of lipophilic ligands diffused through the plasma membrane to the HBD of the receptors. This binding induces conformational changes and homo- or hetero-dimerization of the receptors, which leads to its dissociation from cytoplasmic chaperone proteins i.e. heat shock proteins (Hsp) and exposure of its nuclear localization sequence (NLS). The NLS directs receptor dimers to translocate into the nucleus where they bind to hormone response elements (HRE, also called steroid response elements (SRE)) and activate transcription of target genes [100, 105].

In addition to classical signaling, 'non-classical' signaling by the hormones has been observed. This signaling facilitates a more rapid response to the presence of the hormone, usually within seconds to minutes [100]. This signaling can be initiated by interaction between the ligand and the classical hormone receptor or may not involve the classical receptor but another plasma membrane receptor such as the G protein-coupled receptor (GPCR), GPR 30. This receptor has been shown to interact with estrogen, but whether it does this alone or within a protein complex is not known [106, 107]. A hallmark of the non-classical pathway is that its activity is not affected by the use of steroid antagonists [100].

Estrogen has been shown to activate the surface receptor, EGFR. 17β -estradiol leads to the transactivation of EGFR via the G-protein coupled receptor homolog, GPR30. This process requires the release of HB-EGF and is enhanced not inhibited by the presence of the ER antagonist ICI182780. This transactivation of EGFR was functional as the downstream protein Erk1/2 was phosphorylated [107].

1.7 Sex hormones and genital epithelial cells

The female reproductive tract can be separated into two regions based on their anatomic location: the lower tract consisting of the vagina and ectocervix and the upper tract consisting of the endocervix, endometrium and fallopian tubes. While the lower reproductive tract is mainly covered with stratified squamous epithelium that can be as thick as 25 layers of epithelial cells, the upper tract is lined with a single polarized layer of columnar epithelial cells [108]. The epithelium lining the female reproductive tract undergoes reorganization over the course of the menstrual cycle. In the endometrium, as estrogen levels initially increase there is a gradual thickening of the layer. This thickening is further advanced and attains its maximum thickness as progesterone levels increase. Withdrawal of estrogen and progesterone leads to shedding of the functional layer of the endometrium and initiation of the menstrual cycle [94, 109-112]. All layers of vaginal squamous epithelial cells proliferate as estrogen levels increase. At mid cycle, as estrogen levels decrease and progesterone begins to increase the superficial layers become smaller [94]. Cervical mucus production is also regulated by the menstrual cycle. In the early stages of the cycle (higher estrogen concentration) the cervix secretes a large amount of clear, thin watery mucus. As progesterone concentration increases later in the cycle the quantity of secreted mucus reduces and becomes a cloudy, viscous substance [95].

Both the lower and upper tracts can be targeted by STIs [2, 113]. The vagina and ectocervix undergo continuous sloughing of additional layers providing a strategy to prevent pathogens from establishing an infection and colonizing. Continuous apical junctional complexes between neighboring cells hold the single layer of columnar epithelial cells, in the upper reproductive tract, together. This creates a mechanical barrier

against the passage of pathogens to the underlying cells and tissues. Whether the lower or upper tract is easier for sexually transmitted pathogens to colonize is up for debate, because while the sloughing is a good mechanical deterrent, the lower tract has a larger surface area for pathogens to access [113].

Most sexually transmitted pathogens first encounter the lower reproductive tract and can move upwards to cause disease [114]. The most vulnerable location in the genital epithelium is the ‘transitional or transformation zone’ between the ecto and endo cervix where the squamous epithelium changes to the single layer columnar epithelium [2]. Both the squamous and columnar epithelial cells are responsive to pathogens and their responses can be regulated by the female sex hormones, estrogen and progesterone [108]. In rodents and Rhesus models, progesterone has been shown to increase susceptibility to viral STIs, while estrogen provides protection or decreases the risk of viral infection. This correlates with the abilities of estrogen and progesterone to respectively, thicken or thin the stratified squamous epithelium of the vagina, and by extension strengthen or weaken the epithelial barrier against STIs in these animals. In humans the effect of progesterone appears to be less destructive to the epithelial lining than in animal models [115].

1.8 Sex hormones and sexually transmitted infections

Sexually transmitted infections (STIs) caused by bacteria or protozoa (i.e. gonorrhea, chlamydial infection, syphilis, chancroid) can generally be cured by single dose or short treatments of antibiotics. While these infections occur worldwide among all sexually active groups, there are significantly more infections among young people in developing countries. The duration of these infections generally depends on the length of

time that it takes to obtain treatment. Some infections such as chlamydial and gonococcal infections are often asymptomatic and thus remain untreated and persist for longer periods of time. Importantly, STIs of bacterial pathogens, such as GC can facilitate an increase in the transmission of human immunodeficiency virus (HIV), both its infectivity and susceptibility. Additionally, STIs that cause genital discharge (e.g. gonorrhoea) increase shedding of HIV [116]. With antibiotic resistant strains of these pathogens, like GC, on the rise, these previously curable STIs are now an emerging health crisis [117].

It well known that sex hormones influence our susceptibility to STIs. Brabin *et al.* show that the stage of the menstrual cycle and usage of hormonal contraceptives influence susceptibility to STIs such as GC, Herpes simplex virus (HSV), HIV and chlamydia [114, 115]. Studies in rhesus monkeys show that progesterone implants make the animals more susceptible to simian immunodeficiency virus (SIV), while estrogen protects against it [118, 119]. In addition to regulating epithelial cells, sex hormones are also capable of regulating the immune response against sexually transmitted bacteria and viruses [2]. The FRT secretes antimicrobials, chemokines and cytokines in an effort to protect itself against invading pathogens. These molecules are constitutively produced, but production can be enhanced when pathogens are detected. Production of these protective molecules appears to follow the estrogen cycle, peaking and ebbing at the same time as estrogen levels [120]. This strongly suggests that estrogen stimulates antibody production and cell-mediated immune responses especially in the early stages of an infection. Whether estrogen provides a protective or harmful effect apparently depends on the infecting organism and stage of infection [114]. Murine models of infection of STIs rely on modifying the hormonal environment of the genital tract in order to facilitate

infection, due to the short menstrual cycle of the mouse. Chlamydia and herpes infection models require progesterone treatment [121, 122], while the GC infection model requires estrogen treatment [123]. The multiple regulatory roles of the hormones in the reproductive tract hinder the application of the mouse model in sexually transmitted pathogen research.

Clinical studies have demonstrated a role for hormone contraceptives in STIs. Bacterial vaginosis (BV), which affects from 29% to as much as 50% of the female population, has been shown to be associated with increased acquisition of STIs. Oral contraceptives have been shown to decrease the risk of BV onset and enhance its remission [124, 125]. Initial exposure of hormonal contraceptives increases immunity to human papilloma virus (HPV), however once a persistent infection has been established the protective effect is lost [114]. The role of hormonal contraceptives on HIV infection has been controversial. Most of the cohort studies that involved sex workers from developing countries showed an increase in susceptibility to and accelerated disease progression of HIV coinciding with contraceptive use [126, 127]. However, another study, by Morrison *et al.* in 2007 showed that contraceptive use has no effect on women's susceptibility to HIV. Reanalysis of the statistics in 2010 showed that the injected progesterone only contraceptive, DMPA, but not oral contraceptives, increased HIV susceptibility [128, 129]. Contraceptives up-regulate HIV co-receptors in the female genital tract [130]. The majority of contraceptive chlamydia studies show that oral contraceptive use increases the risk of infection [131, 132].

Studies of hormonal effects on GC infection on the other hand are less consistent. Most of the cross sectional studies show no association of oral contraceptives with GC

infections [131]. Cohort studies that take sexual behavior into account show that oral contraceptives increase the risk of contracting gonorrhea [132] and asymptomatic female patients have increased blood progesterone levels [133]. Contrary to those reports, a study by Gursahaney *et al.* show that oral contraceptive users actually had a reduced risk of contracting gonorrhea from male partners diagnosed with the infection. This suggests a protective role for hormones in gonorrhea transmission [134]. It has been clinically shown that gonococcal PID and DGI occur more frequently during menses, implying that hormone levels may play a role in gonorrhea infectivity. Additionally, GC growth is enhanced in menstrual blood that is iron rich, and use of contraceptives reduces blood flow, thus decreasing the iron supply in the genital tract. How the regulatory roles of the hormones on genital epithelial cells and mucosal immunity impact GC transmission and infectivity is largely unknown. The combination of clinical and basic research is required for a comprehensive understanding of GC infection in this unique, hormonally controlled environment.

1.9 Rationale

The goal of my PhD study is to obtain a better understanding of the interaction of *Neisseria gonorrhoeae* with polarized epithelial cells during the infection process and the impact of the female sex hormones on this interaction. My study is a part of the collaborative interest of the Song and Stein labs in the progression of GC infection in women. The high infection rate and serious sequelae of the infection in young women is a significant public health concern. The invasive infection in women, which can lead to PID, is associated with the transmigration of GC across the epithelium lining the female

reproductive tract and invasion of GC into subepithelial tissue. *N. gonorrhoeae* is an obligate human pathogen and currently there is no good animal model for my study. An available mouse model requires high doses of estrogen to maintain a transient infection and therefore is not suitable for investigating how human sex hormones affect the initiation of the infection and progression of the disease. Most previous *in vitro* studies have focused on the interaction of GC with non-polarized epithelial cells. How GC interact with polarized epithelium lining the upper reproductive tract has not been well studied. I have developed a polarized model of two epithelial cell lines, an endometrial epithelial cell line HEC-1-B and a colonic epithelial cell line T84, both of which have been extensively used in the pathogenesis research field. The GC MS11 pili positive, opa positive strain was derived from a male patient. Using these polarized epithelial cell lines and GC strain, I have tested my hypothesis that *GC cause activation of EGFR, leading to disruption of the junctional complex and increased GC transmigration across the polarized monolayer and this transmigration is enhanced by the presence of estrogen and progesterone.*

1.10 Aims

1.10.1 Aim 1

This aim was designed to examine the hypothesis that *N. gonorrhoeae* affects the junctional complex in a manner that requires activation of host cell surface receptor(s). I show that GC disrupt the localization of both tight junction and adherens junction proteins and that epidermal growth factor receptor (EGFR) activation is involved. Junction disruption leads to increased GC transmigration.

1.10.2 Aim 2

This aim was designed to examine role of the female sex hormones, estrogen and progesterone, on *N. gonorrhoeae* pathogenesis. I show that while the hormones have no effect on the early stages of GC pathogenesis, adherence and invasion, they facilitate an increase in GC transmigration.

Chapter 2: *Neisseria gonorrhoeae* Breaches the Apical Junction of Polarized Epithelial cells for Transmigration by Hijacking EGFR

2.1 Introduction

Neisseria gonorrhoeae causes gonorrhea, a common sexually transmitted infection (STI). This gram-negative, obligate human pathogen causes different disease sequelae in men and women. The highest reported cases of gonorrhea are among teenage girls and young women [6]. Since most gonococcal (GC) infections in women are asymptomatic, the infections remain undiagnosed and untreated, thus predisposing women to pelvic inflammatory disease (PID) and disseminated gonococcal infection (DGI), which can lead to infertility and arthritis, respectively [3, 6, 9]. Clinical studies show an association of GC infection with an increased risk of HIV infection [135, 136], highlighting the significance of GC infection in public health. No vaccine has been successfully developed due to a lack of understanding of the cellular mechanism underlying the interaction of this bacterium with the mucosal surface of the female genital tract.

In women, the primary target of GC is the epithelial cell monolayer that lines the reproductive tract [4, 7]. The interaction of GC with epithelial cells has been extensively studied, primarily using non-polarized epithelial cell lines or organ culture. Initiation of colonization is mediated by pili, which bind to host surface receptors on columnar endocervical epithelial cells. Subsequent contraction of pili brings the bacteria close to the epithelial cells [4, 8, 137], allowing GC to establish a more intimate attachment to the host membrane via opacity proteins (Opa) and lipooligosaccharides (LOS) [3, 4]. In

epithelial cells, these interactions induce a variety of signaling cascades, including calcium flux, phosphoinositide 3-kinase (PI3K), phospholipase C (PLC) and the mitogen-activated protein kinase (MAPK) Erk, leading to actin reorganization, microvillus elongation and the subsequent engulfment of GC [4, 28]. We have shown that the interaction of GC with human endometrial epithelial cells, HEC-1-B, increases the phosphorylation of epidermal growth factor receptor (EGFR) by triggering the expression and surface cleavage of EGFR ligands. This GC-induced EGFR trans-activation is required for GC invasion into non-polarized HEC-1-B cells. In addition to EGFR phosphorylation, apical inoculation of GC leads to a redistribution of EGFR from the basolateral surface of polarized HEC-1-B cells to GC adherent sites at the apical surface [90]. Cleavage of these EGFR ligands generally occurs through activation of matrix metalloproteinases (MMPs) or the related a disintegrin and metalloproteinase (ADAM) [138-140], and inhibition of this cleavage activity reduce the invasion of GC into host epithelial cells [89].

In addition to attachment and invasion, GC are capable of transmigrating across polarized epithelial cells cultured *in vitro* [17, 34, 35, 141]. Subepithelial bacteria have been found in organ culture models and clinical samples from patients [13, 142, 143]. This suggests that GC transmigration is associated with the pathogenicity of the bacteria, however the cellular mechanism underlying GC transmigration is largely unknown. Based on the invading capability of GC, it has been proposed that GC transmigrate via an intracellular pathway, in which apically internalized GC traverse to and exit from the basolateral membrane of epithelial cells [13, 35].

The single layered endocervical columnar epithelial cells have been shown to be the preferred tissue target for GC infection. The monolayer of epithelial cells on the mucosal surface is held together via apical junction complexes formed continuously between neighboring cells. The apical junction complexes seal the paracellular space between epithelial cells, creating a physical barrier against pathogen movement via the space between cells. The apical junction also provides a barrier against the lateral movement between the apical and basolateral membrane, generating and maintaining the polarized distribution of proteins and lipids in the apical or basolateral membrane and their distinct physiological functions [46, 51, 144]. The actin cytoskeleton provides scaffolding supports for microvilli and the apical junction [45, 52, 57, 60, 145]. The apical junction contains the tight junction and adherens junction [49, 57]. The tight junction consists of transmembrane proteins such as occludin and claudins [46, 144] and associated protein ZO-1 that links the tight junction to the actin cytoskeleton [45, 57]. The adherens junction is formed through calcium dependent trans-homophilic interaction of E-cadherin on neighboring cells [146, 147] and is required for the assembly of the tight junction. Therefore, removing extracellular calcium leads to the disassembly of the apical junction [78]. The apical junction is a dynamic structure and its barrier function as well as assembly and disassembly are regulated by external and internal cell signaling through junction-associated proteins, such as β -catenin [51, 144, 148, 149]. Signaling mediated by surface receptors, such as EGFR [79, 87], induces the phosphorylation of β -catenin, which causes the dissociation of β -catenin from the junctional complex and the actin cytoskeleton [58, 89, 150, 151]. Endocytosis and lysosomal degradation of the junctional proteins can also lead to disassembly of the apical junction [71, 152, 153].

In order to establish infection, GC must attach to epithelial cells. Invasive diseases may require GC invasion into and/or transmigration across polarized epithelium. While the interaction of GC with non-polarized epithelial cells has been extensively studied, how GC interaction with polarized epithelial cells impacts the epithelial barrier has not been fully investigated. Many mucosal bacterial pathogens, including *Clostridium difficile*, *Escherichia coli*, *Vibrio cholerae*, *Helicobacter pylori*, *Clostridium perfringens*, *Listeria monocytogenes* and *Bacteroides fragilis*, have developed means to weaken the epithelial barrier by directly or indirectly regulating the apical junction. The interaction of these pathogens with polarized epithelial cells disrupts the integrity of the junctional complex and increases epithelial permeability, which facilitate the passage of pathogens through the paracellular space [46, 91]. Furthermore, *N. meningitidis*, the other pathogenic species of *Neisseria*, transmigrates across endothelial cells by disrupting VE-cadherin-based intercellular junctions [92, 93].

In this study, we examine the interaction of GC with polarized epithelial cells. We observed that apical inoculation of GC induces the disassembly of the apical junction, which is concurrent with a decrease in the barrier function of the apical junction against the lateral movement between the apical and basolateral membrane. GC-induced junction disassembly depends on the kinase activity of EGFR. Disrupting the apical junction using the calcium chelator, EGTA, increases GC transmigration. Inhibition of GC-induced junction disassembly by an EGFR kinase inhibitor significantly reduces GC transmigration. These results provide the first evidence that GC can negatively regulate the apical junction of polarized epithelial cells for its transmigration via activating EGFR.

2.2 Materials and Methods

2.2.1 Epithelial Cells

Human endometrial adenocarcinoma cell line, HEC-1-B cells (ATCC# HTB-113, Manassas VA, USA), were maintained in Eagles MEM, alpha medium supplemented with 10% heat inactivated fetal bovine serum (FBS). Human colorectal carcinoma cell line, T84 cells (ATCC# CCL-248), were maintained in Dulbecco's modified Eagle's medium:Ham F12 (1:1) supplemented with 7% heat inactivated FBS. Cells were maintained at 37 °C and 5% CO₂. Cells were seeded at 6x10⁴ (6.5 mm diameter transwell) or at 1x10⁵ (24mm diameter transwell) per transwell (3 µm pore size, polyester transwells inserts, Corning, Lowell, MA, USA) and cultured for ~10 days until transepithelial electrical resistance (TER) reached ~400 Ω (HEC-1-B) and ~2000 Ω (T84). TER was measured using a Millicell ERS volt-ohm meter (Millipore, Bedford, MA, USA).

2.2.2 Neisseria Strains

N. gonorrhoeae strain MS11 that expressed both pili and Opa (Pil⁺ Opa⁺) were used. Gonococci (GC) were grown on GC media base plates with 1% Kellogg's supplement (GCK) [154] for 15–18 h before inoculation. Pil⁺ Opa⁺ colonies were acquired based on their morphology using a dissecting light microscope. Bacteria were placed in suspension and the concentration determined using a spectrophotometer. An OD of 1 represented 10⁹ GC. Gentamicin killed GC were generated by incubating the bacteria with 100 µg/ml gentamicin sulfate for 4 h at 37 °C and then overnight at 4 °C. GC were inoculated with epithelial cells at MOI 10:1 (viable GC) and 20:1 (killed GC).

2.2.3 Immunofluorescence analysis

Cells were serum starved overnight, pre-treated with or without the EGFR inhibitor AG1478 (10 μ M, Calbiochem, San Diego, CA, USA) for 2 h, and incubated with GC in the presence or absence of the inhibitor for 6 h. Cells were washed and fixed using a pH shift method [155], permeabilized, and stained with anti-ZO-1 (BD Bioscience, Bedford, MA, USA), anti-occludin (Invitrogen, Camarillo, CA, USA), anti- β -catenin (Millipore, Temecula, CA, USA), and anti-GC [156] antibodies. Cells were analyzed by confocal fluorescence microscopy (Zeiss LSM 510 or 710, Carl Zeiss Microscopy LLC, Thornwood, NY, USA). Z-series of images were obtained in 0.5 μ m slices from the top to the bottom of cells, and three-dimensional (3D) composites obtained. Fluorescence intensity profiles were generated using the ImageJ software. The percentage of cells showing discontinuous staining of junctional proteins was determined by visual inspection of multiple image slices. The redistribution of β -catenin from the apical junction to the cytoplasm was quantified by the fluorescence intensity ratio (FIR) of β -catenin at the cell-cell junction to that at the cytoplasm using fluorescence intensity profiles generated by the ImageJ software.

2.2.4 Immunoprecipitation and immunoblotting analyses

Polarized epithelial cells apically incubated with bacteria for varying periods were lysed using RIPA buffer [90]. The cell lysates were incubated with protein A Sepharose beads (GE Healthcare, Piscataway, NJ, USA) and anti- β -catenin (Millipore) or anti-phosphotyrosine mAb (4G10) (Millipore) Abs. Immunoprecipitates were resolved by SDS/PAGE gels and analyzed using western blotting. For anti- β -catenin

immunoprecipitation, the blots were probed for phosphotyrosine using 4G10 mAb to determine phosphorylated β -catenin. For phosphotyrosine immunoprecipitation, the blots were probed for EGFR (Santa Cruz, CA, USA). The total levels of β -catenin and EGFR in the cell lysates were detected by western blotting using specific antibodies. The blots were imaged using Fujifilm's LAS-3000 (Valhalla, NY, USA) and quantified by Fujifilm's MultiGuage software.

2.2.5 Functional analyses of the apical junction

To determine the effect of GC on the fence function of the apical junction against lateral mobility between the apical and basolateral membrane, HEC-1-B cells were seeded at 1×10^5 on the underside of transwells and cultured for 10 days until TER reached the optimal level ($\sim 400\Omega$). GC were added to the apical surface and incubated for 4 h. Time lapse xz images were acquired in the presence of the CellMask dye (5 $\mu\text{g/ml}$, Invitrogen), (excitation and emission wavelengths were 554 and 567 respectively), to mark the apical membrane for 30 min using the Leica TCS SP5 X confocal microscope (Leica Microsystems, Inc, Buffalo Grove, IL, USA). Over the course of the imaging the cells were enclosed in a humidifying chamber at 37 °C and exposed to 5% CO₂. The fluorescence intensity ratio of the CellMask dye at the apical to basolateral membrane was determined.

To determine the effect of GC on the gate function of polarized epithelial cells against the diffusion of molecules through the paracellular spaces, cells were incubated with GC and Lucifer Yellow (500 μM , Sigma, Saint Louis, MO, USA) or FITC (50 μM , Acros Organics, Geel, Belgium) in the apical chamber for 6 h. The fluorescence intensity

of Lucifer Yellow (excitation 425nm, emission 528nm) and FITC (excitation 490nm, emission 525nm) in the apical and basolateral media was determined using a fluorometer. All readings were taken at room temperature.

2.2.6 GC invasion and transmigration assays

Polarized epithelial cells were incubated apically with GC for 6 h at 37 °C. When the EGFR inhibitor was used, cells were pretreated with AG1478 (10 µM) for 2 h and incubated with GC in the presence of the inhibitor. Media from the basal compartment was collected and plated onto GCK to determine the number of transmigrated bacteria. Cells were exposed to gentamicin (100 µg/ml) for 2 h to kill extracellular bacteria, then lysed and plated to determine invaded bacteria.

2.2.7 Statistical analysis

Statistical significance was assessed using the Student t-test by Prism software (GraphPad Software, San Diego, CA). *p* values were determined in comparison to controls.

2.3 Results

2.3.1 Preferential localization of live gonococci at the cell-cell junction of polarized epithelial cells.

To investigate the interaction of gonococci (GC) with polarized epithelial cells, we used confocal microscopy to analyze the distribution of GC on HEC-1-B and T84 cells that have relatively low and high polarity respectively based on their TER readings.

The polarization of these cells was confirmed by a measured increase in the transepithelial electric resistance (TER) and the visualization of the polarized distribution of apical junction proteins. After apical inoculation of GC (live or gentamicin killed at MOI of 10 or 20, respectively) for 6 h, the polarized epithelial cells were stained for the apical junctional protein ZO-1 and for GC, and analyzed using three-dimensional (3D) confocal fluorescence microscopy. The percentage of GC clusters located in the vicinity of the cell-cell junction marked by ZO-1 was determined by visual inspection. The data show that approximately 80% of live GC clusters, but only 40-50% of killed GC clusters, localized at the cell-cell junction of both HEC-1-B and T84 cells (Fig. 7). This result indicates that GC preferentially localize at the apical cell-cell junction in polarized epithelial cells despite their different levels of polarity and tissue origins, and this localization is more efficient when the bacteria are viable.

2.3.2 GC inoculation disrupts the continuous apical junctional complexes between polarized epithelial cells

The preferential cell-cell junctional location of GC implicates a possible impact of GC on the apical junction of polarized epithelial cells, similar to phenomena observed in other mucosal bacterial pathogens [157-163]. To examine the effects of GC on the distribution of apical junctional proteins, polarized HEC-1-B (Fig. 8) and T84 (Fig. 9) cells were incubated with GC in the apical compartment for 6 h and stained for the apical junctional protein, ZO-1 or occludin. Fluorescence intensity (FI) profiles of ZO-1 and occludin were generated from images acquired by confocal microscopy. In the absence of GC, ZO-1 (Fig. 8Aa, 8Ad) and occludin (Fig. 8Ba, 8Bd) staining continuously

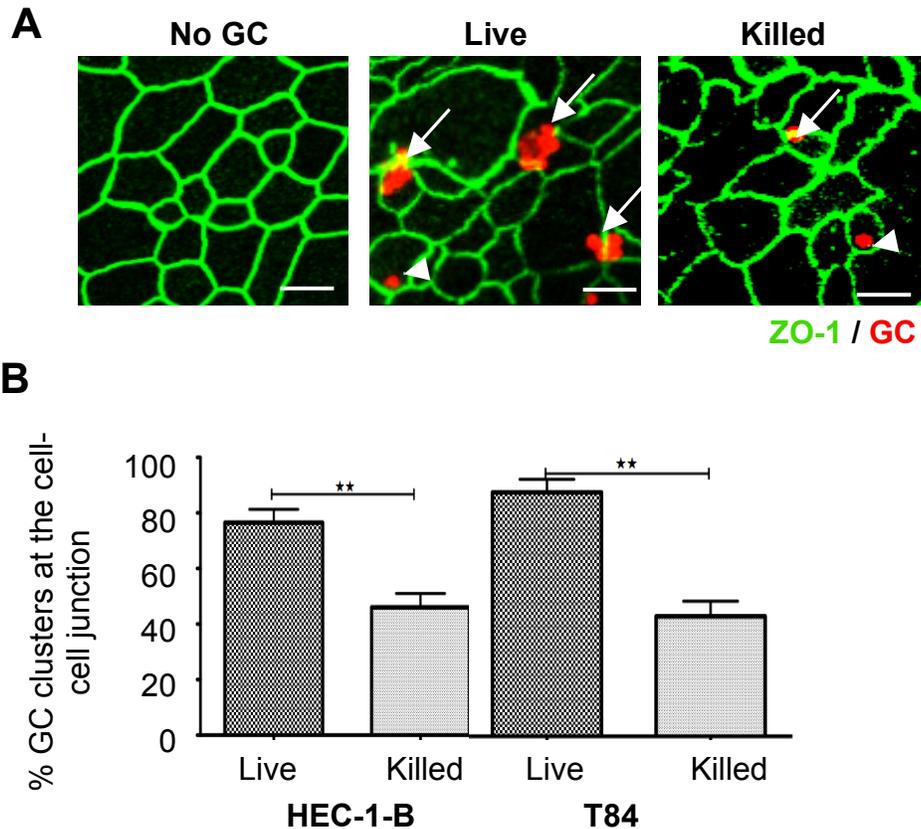


Figure 7. Live GC preferentially localize at the cell-cell junction of polarized epithelial cells. Polarized HEC-1-B and T84 cells were apically inoculated with live or gentamicin killed GC ($P^{+}O^{+}$ MS11) at a MOI of 10 and 20, respectively, for 6 h. Cells were fixed, permeabilized and stained for ZO-1 and GC, and analyzed using confocal microscopy. The number of GC clusters localized at (long arrows) or not at (arrow head) the cell-cell junction marked by ZO-1 in HEC-1-B and T84 cells was quantified by visual inspection. Shown are representative images (A) and the average percentages (\pm S.D.) of GC clusters at the cell-cell junction (B) from three independent experiments. Scale bar, 10 μ m. **, $p \leq 0.01$.

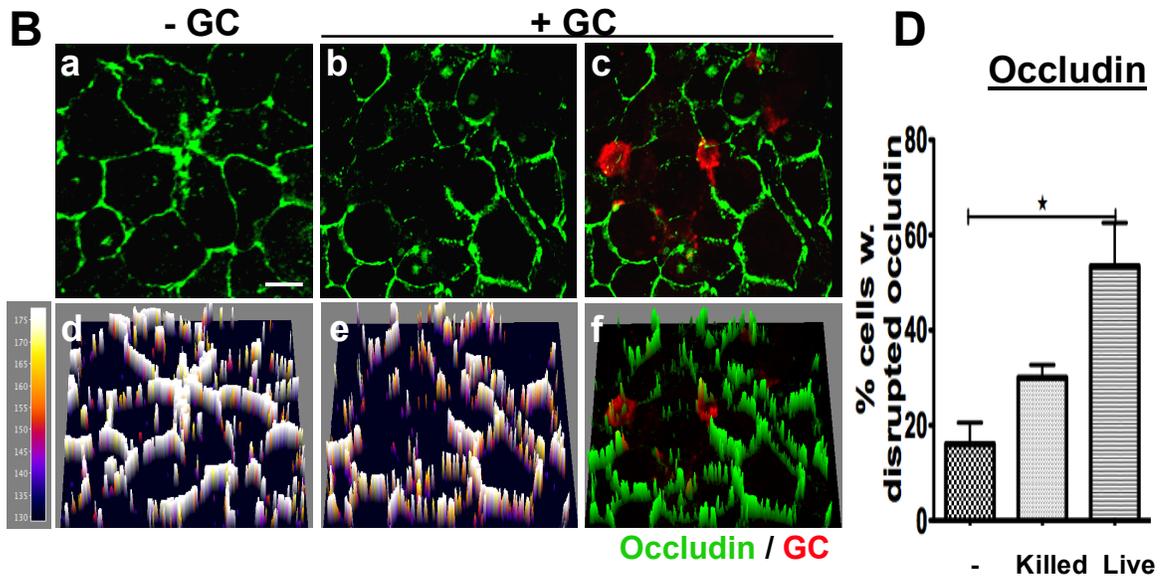
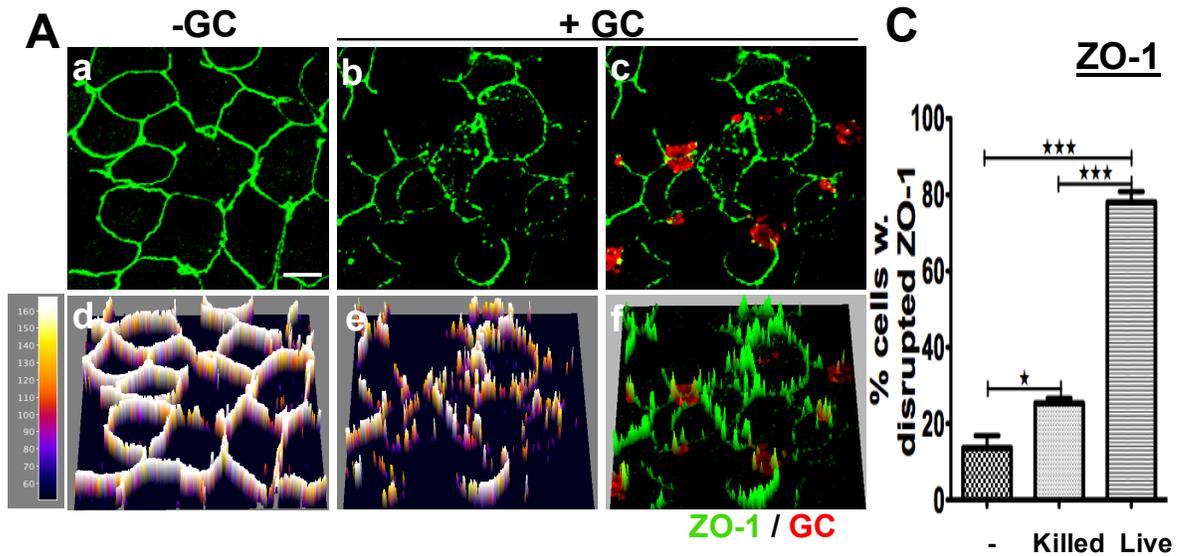


Figure 8. Viable but not killed GC disrupt the continuous apical junction location of ZO-1 and occludin in polarized HEC-1-B cells. Polarized HEC-1-B cells were incubated with media only (a and d), live GC (b, c, e, and f) or gentamicin killed GC in the apical compartment for 6 h. Cells were fixed and stained for ZO-1 (A) or occludin (B) and GC, and then analyzed using confocal microscopy. Shown are representative images (composites of 1 μm slices) and their fluorescence intensity profiles. Cells with disrupted ZO-1 (C) and occludin (D) peripheral staining were quantified by visual inspection, and the average percentages (\pm S.D.) from three independent experiments are shown. Scale bar, 5 μm .

Figure 9. GC inoculation disrupts the continuous apical junction location of ZO-1 and occludin in polarized T84 cells. Polarized T84 cells were incubated with media only (a) or GC (b-c) in the apical compartment for 6 h. Cells were stained for ZO-1 or occludin and GC and analyzed using confocal microscopy. Shown are representative images of ZO-1 (A) and occludin (B) and their fluorescence intensity profiles (d-e). Cells with disrupted ZO-1 (C) and occludin (D) peripheral staining were visually quantified, and the average percentages (\pm S.D.) from three independent experiments are shown. Scale bar 10 μ m. ** , $p \leq 0.01$.

resulted in significant redistribution of junctional complex proteins, with 78% and 53% of HEC-1-B cells showing discontinuous ZO-1 and occludin staining respectively. However, gentamicin killed GC that can attach to but not invade into non-polarized epithelial cells [156] had no significant effect on ZO-1 (Fig. 8C) and occludin staining (Fig. 8D). While the level of disruption of the apical junction in T84 cells appeared to be lower than that observed in HEC-1-B cells, GC interaction still increased the percentage of cells with discontinuous staining of ZO-1 and occludin from 0 to 18% and 33% respectively (Fig. 9). These results indicate that the inoculation of viable GC, but not killed GC, induces the disassociation of ZO-1 and occludin from the apical junction of polarized epithelial cells, suggesting a capability for GC to induce the disassembly of the apical junction during infection.

2.3.3 GC inoculation decreases the fence but not gate function of the apical junction

GC-induced redistribution of the apical junctional proteins potentially affects the functionality of the junctional complex. The apical junction performs two significant roles in polarized epithelial cells. The ‘fence’ function prohibits proteins and lipids in the apical and basolateral membrane from laterally moving into the other side, thereby maintaining the functional polarity of two surfaces on epithelial cells. The ‘gate’ function controls the paracellular permeability of epithelial cells, preventing mucosal pathogens from crossing through the paracellular space between epithelial cells. To examine the fence function, polarized HEC-1-B cells were grown on the underside of transwells and apically incubated with live GC at MOI of 10 for 4 h. The CellMask dye was added to the

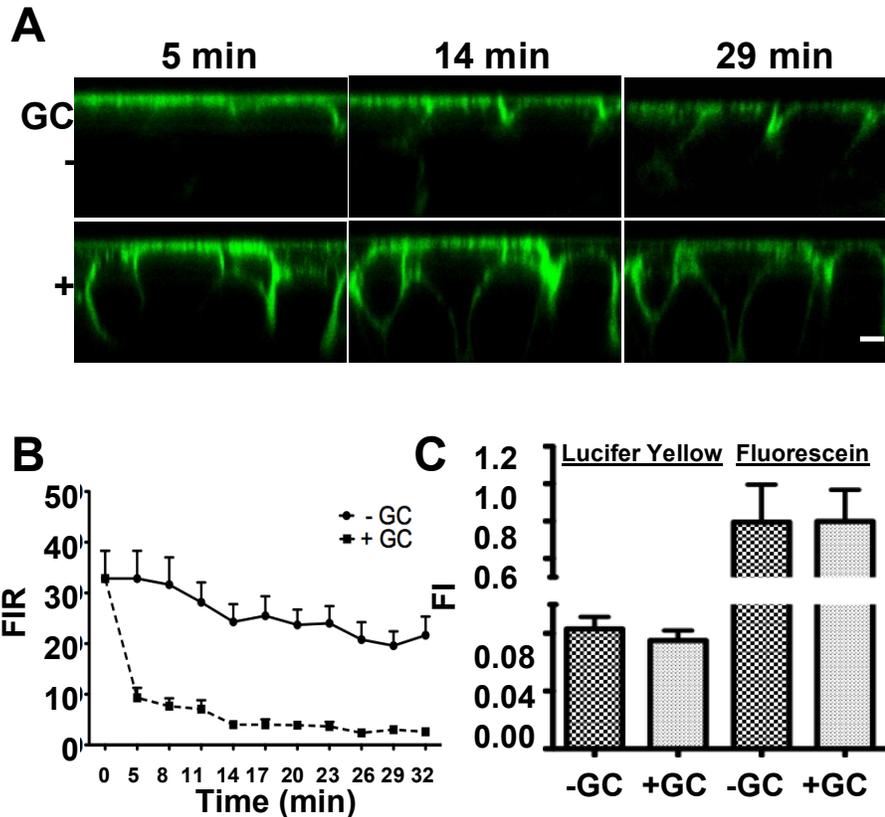


Figure 10. GC inoculation increases the lateral mobility between the apical and basolateral membrane, but not the permeability of the apical junction in polarized epithelial cells. (A-B) Polarized HEC-1-B were incubated with media alone (-) or GC (+) for 4 h. Cells were then apically exposed to the CellMask membrane dye, and live time lapse images were acquired using a confocal microscope. The apical to basolateral fluorescence intensity (FI) of the CellMask dye over time was determined (B). (C) Epithelial cells were incubated with or without GC for 6 h in the presence of Lucifer yellow or FITC dye. The fluorescence intensity of Lucifer yellow or FITC in the apical and basal compartments was determined using a luminometer. The basal compared to apical FI of Lucifer yellow and FITC, respectively, was determined. Shown are the represent images and the averages values (\pm S.D.) from three independent experiments. Scale bar, 5 μ m.

apical chamber to stain the apical membrane exclusively, and cells were analyzed by time lapse microscopy. An increase of the CellMask staining in the basolateral surface was used as the indication of a decrease in the fence function of the apical junction. To quantify the lateral movement of the dye from the apical to the basolateral surface, we determined the Fluorescence Intensity Ratio (FIR) of the dye in the apical surface to that in the basolateral surface. In the absence of GC, the majority of the apically added CellMask dye remained at the apical region of epithelial cells with the FIR at ~33. This FIR decreased slowly over time and reduced to ~22 by 30 min post CellMask staining (Fig. 10A-B). In the presence of GC there was a rapid decrease in the CellMask dye in the apical membrane, with a concomitant increase in the CellMask dye in the basolateral region, leading to a reduction in the FIR from 33 to 9 within the first 5 min of staining (Fig. 10A-B). The FIR further decreased over time and by 30 min, the FIR in GC-infected epithelial cells was reduced below 3 (Fig. 10A-B). These results show that GC inoculation significantly increases the lateral mobility from the apical to basolateral membrane, suggesting that GC induce a significant reduction in the fence function of the apical junction.

The effect of GC on the gate function of the apical junction was determined by measuring the permeability of epithelial monolayers to Lucifer yellow and fluorescein, which are dyes with small molecular masses. These dyes represent small molecules that can pass between the paracellular space of neighboring cells, a process/movement regulated by the apical junctional complex. Polarized HEC-1-B cells were incubated with the Lucifer yellow or fluorescein apically in the absence or presence of GC for 6 h. The FIR of the dye in the basal to that in the apical chamber was determined and used as a

quantitative measure of the apical to basal permeability. We found that GC inoculation had no significant effect on the amount of either lucifer yellow or fluorescein diffusion to the basolateral medium (Fig. 10C). Consistent with this finding, we did not detect significant decreases in TER after 6 h GC incubation (data not shown). Disrupting the apical junction with the calcium chelator EGTA significantly increased the amount of the dyes in the basal medium (data not shown). These results indicate that GC inoculation reduces the fence function of the apical junction, but it does not significantly alter the permeability of the epithelium.

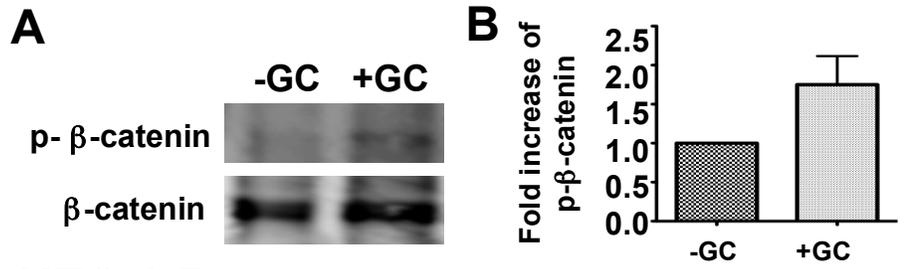
2.3.4 Phosphorylation and redistribution of β -catenin in GC-infected epithelial cells

The apical junction undergoes rapid assembly and disassembly in response to internal and external signals [144]. β -catenin provides a link between the apical junction and cellular signaling [51, 56, 164, 165]. To understand how GC regulate the apical junction, we examined the effects of GC inoculation on the phosphorylation and cellular distribution of β -catenin. Polarized HEC-1-B cells were incubated with or without GC in the apical chamber for 4 h. Phosphorylated β -catenin was detected and quantified using immunoprecipitation and western blot analysis. We found that the presence of GC significantly increased the phosphorylation level of β -catenin without altering its total protein level (Fig. 11A-B), suggesting that GC stimulates β -catenin phosphorylation. We used immunofluorescence microscopy to analyze the cellular distribution of β -catenin. The disassociation of β -catenin from the apical junction was quantified using the FIR of β -catenin at the cell-cell junction to that in the cytoplasm. β -catenin was concentrated at the periphery of cells in the absence of GC in both polarized HEC-1-B and T84 cells

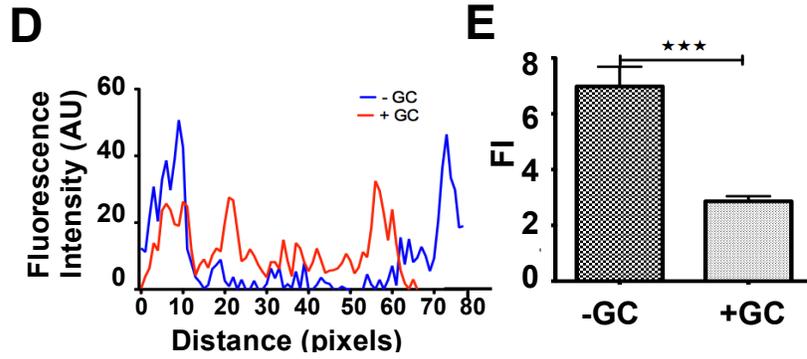
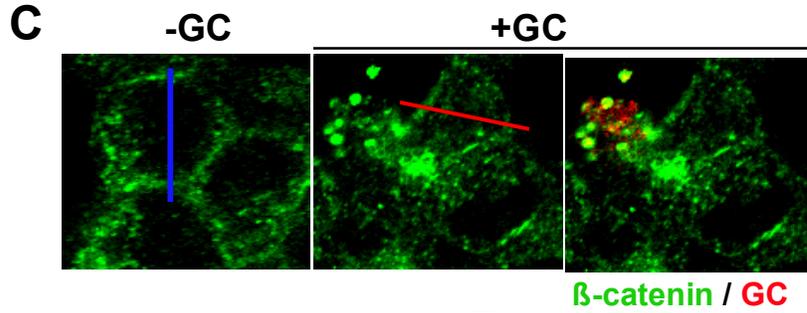
(Fig. 11C-H). When exposed to GC, there were increases in the cytoplasmic levels with parallel decreases in the junctional level of β -catenin, leading to reductions in the junction to cytoplasm FIR of the β -catenin staining in both polarized HEC-1-B and T84 cells (Fig. 11C-H). Concurrent with the redistribution, a portion of β -catenin staining appeared to be colocalized with GC clusters (Fig. 11C and 11F), suggesting a recruitment of β -catenin to GC adherent sites. In contrast, incubation with gentamicin-killed GC for the same length of time did not affect the cellular distribution of β -catenin (Fig. 14C and 14D). These results demonstrate that the presence of GC induces the phosphorylation and redistribution of β -catenin from the apical junction to the cytoplasm, and suggest that GC regulate the apical junction by modulating the activity of β -catenin.

2.3.5 GC inoculation does not change the cellular level of junctional proteins

A common mechanism underlying the disassembly of the apical junction is the endocytosis and lysosomal degradation of junctional proteins. To determine if GC trigger such a mechanism, we compared the cellular levels of the junctional proteins, occludin and ZO-1, in polarized HEC-1-B cells with and without 6 h GC incubation. Using western blot analysis, we did not detect significant differences in the total protein levels of ZO-1 and occludin in GC inoculated epithelial cells in comparison to those without GC (Fig. 12). This result suggests that GC inoculation does not lead to a significant degradation of junction proteins, and that degradation of junction proteins is unlikely to be the mechanism for GC-mediated regulation of the apical junction.



HEC-1-B



T84

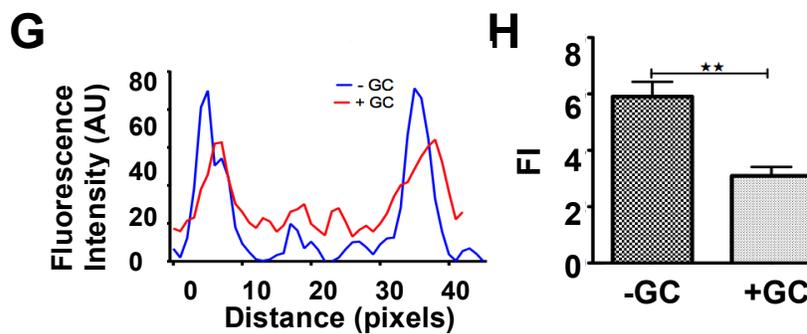
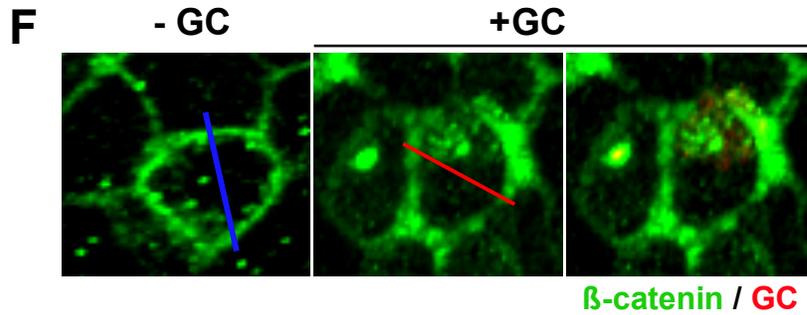


Figure 11. GC inoculation induces the phosphorylation and redistribution of β -catenin from the apical junction to the cytoplasm. (A-B) Polarized HEC-1-B cells were incubated apically with or without GC for 4 h. Cells were lysed and subjected to immunoprecipitation using β -catenin-specific antibodies. Immunoprecipitates were analyzed by SDS-PAGE and Western blot probing for phosphotyrosine. The blot was quantified by densitometry to determine the p β -catenin fold increase over no GC control. (C-H) Polarized HEC-1-B (C-E) and T84 (F-H) cells were incubated with or without GC apically for 6 h. Cells were stained for β -catenin and GC and analyzed using confocal microscopy. Fluorescence intensity profiles along a line crossing cells (D and G) were generated to determine the β -catenin FI at the membrane compared to the cytoplasm (E and H). Shown are representative blots, images, fluorescent intensity profiles of the representative images, and the averages ratios (\pm S.D.) from three independent experiments. Scale bar, 5 μ m. ***, $p \leq 0.001$. **, $p \leq 0.01$.

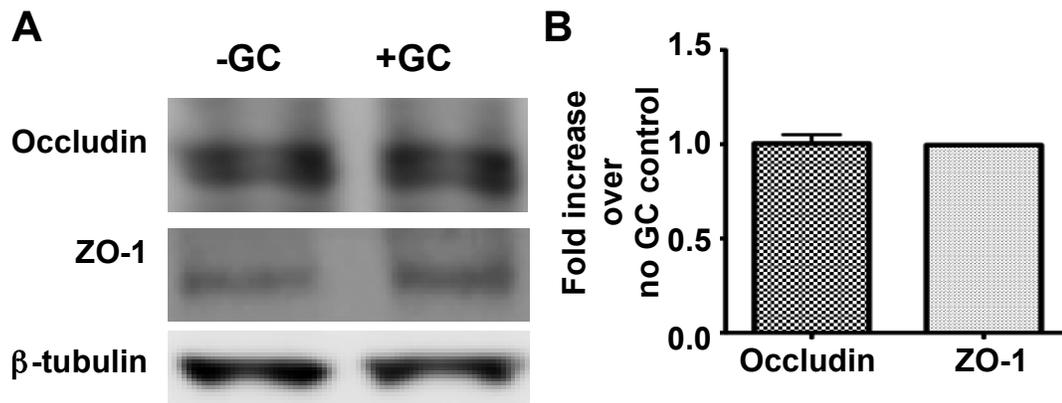


Figure 12. GC inoculation does not change the cellular levels of junctional proteins. HEC-1-B cells were lysed and analyzed by SDS-PAGE and western blot, probing for junctional proteins, ZO1, and occludin, and β -tubulin as the loading control. Shown are representative blots (A) and the average densitometry values (\pm S.D.) from three independent experiments (B).

2.3.6 Disrupting the apical junction with EGTA increases GC transmigration across polarized epithelial cells

The apical junction is essential for the mucosal epithelium's barrier function. To investigate if junctional regulation is important for GC infection, we determined if disruption of the apical junction by EGTA has any effect on GC invasion and transmigration. EGTA is a Ca^{2+} chelator that induces the disassembly of the apical junction by inhibiting Ca^{2+} -dependent trans-homophilic interaction of E-cadherin on neighboring epithelial cells [78]. Polarized HEC-1-B cells were pre-treated with EGTA [5 mM] in both the apical and basal compartments for 10 min, and then the EGTA was removed by washing before GC inoculation in the apical compartment. EGTA pre-treatment dramatically reduced TER and caused a complete loss of the polarized distribution of ZO-1, but did not affect the viability of GC (data not shown), confirming the efficacy of EGTA in junction disruption. After the 6 h incubation with GC, the CFU of GC recovered from the basal medium was increased 100 fold in EGTA-treated HEC-1-B cells, compared to cells without EGTA treatment (Fig. 13A). However, EGTA treatment did not significantly change the level of GC invasion into HEC-1-B cells (Fig. 13B). Therefore, disrupting the apical junction increases GC transmigration across polarized epithelial monolayers, but not invasion into the cells.

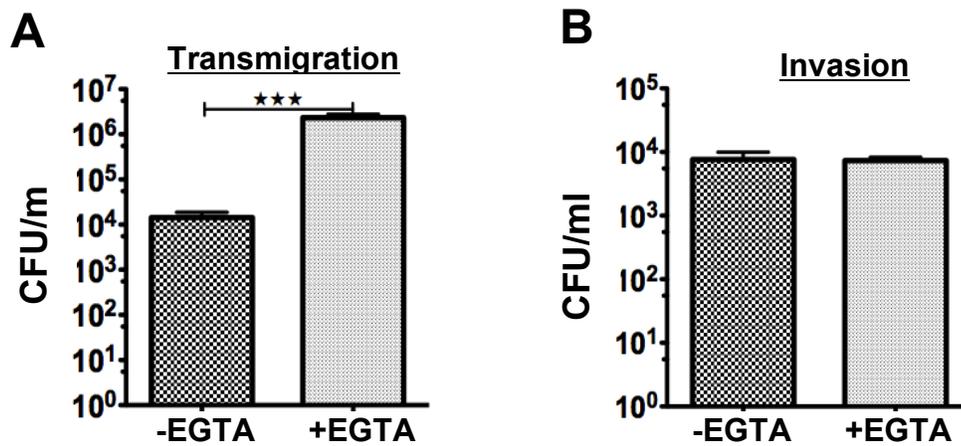


Figure 13. Disrupting the apical junction by EGTA increases GC transmigration. Polarized HEC-1-B cells were pretreated with or without EGTA (5 mM) apically and basally for 10 min. Cells were then washed and inoculated with GC at a MOI of 10 for 6 h. The basal media was collected and plated to determine the transmigrated GC. Cells were also washed and incubated with gentamicin, then lysed and the bacteria collected and plated to determine invaded GC. Shown are the average values (\pm S.D.) from three independent experiments. ***, $p \leq 0.001$.

2.3.7 GC-induced β -catenin redistribution depends on the kinase activity of EGFR

The increased phosphorylation level and redistribution of the junction signaling connector, β -catenin, in GC inoculated epithelial cells suggest that GC potentially regulate the apical junction via activating host signaling cascades. Based on previous findings that GC inoculation increases EGFR phosphorylation [90] and that EGFR activation has been shown to lead to β -catenin phosphorylation [79, 89, 150], we hypothesized that GC-induced redistribution of β -catenin is related to EGFR activation.

We examined whether GC inoculation impacts EGFR phosphorylation in polarized epithelial cells and if the EGFR kinase inhibitor, AG1478, has any effect on GC-induced redistribution of β -catenin. The phosphorylation of EGFR was determined by immunoprecipitation and western blot. Similar to our previous observations in non-polarized cells [90], the level of phosphorylated EGFR in polarized HEC-1-B cells was increased after cells interacted with GC for 4 h, compared to media only controls (Fig. 14A-B). To inhibit EGFR activation, polarized cells were pre-treated for 2 h with the EGFR kinase inhibitor AG1478 and then incubated with GC in the presence of the inhibitor for 6 h. Treatment with the EGFR inhibitor significantly reduced GC-induced redistribution of β -catenin from the membrane to the cytoplasm in both polarized HEC-1-B and T84 cells, increasing the membrane to cytoplasm FIR of β -catenin from ~ 3 back to 5 (Fig. 14C-D). Additionally, the treatment of the EGFR inhibitor reduced the colocalization between GC microcolonies and β -catenin for both polarized HEC-1-B and T84 cells (Fig. 14E and 14F). The inhibitory effect of the EGFR kinase inhibitor on GC-induced redistribution of β -catenin and β -catenin colocalization with GC microcolonies

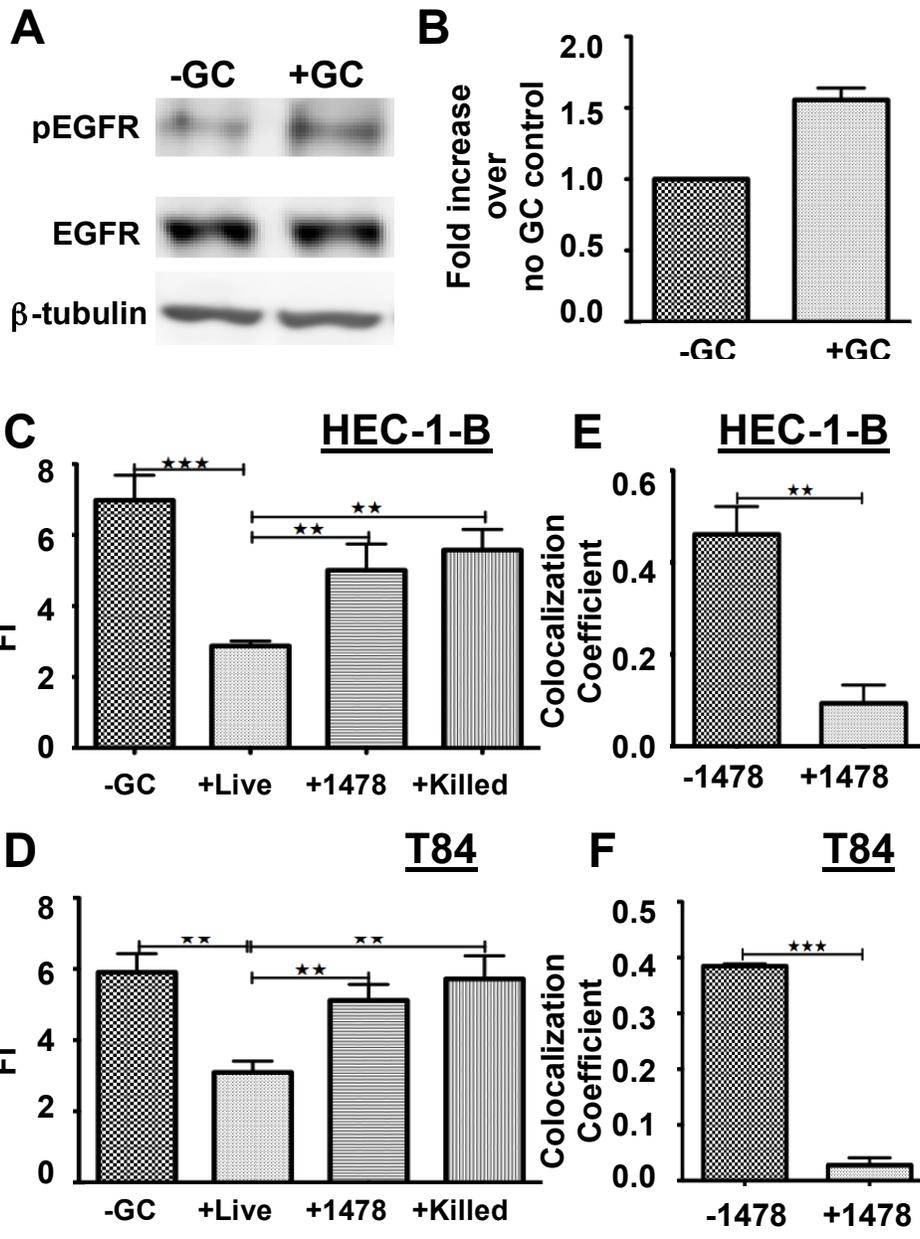


Figure 14. GC inoculation induces the phosphorylation of EGFR, which is required for GC-induced redistribution of β -catenin. (A-B) Polarized HEC-1-B cells were incubated with or without GC for 4 h and then lysed. Cell lysates were subjected to immunoprecipitation using phosphotyrosine-specific antibody. Immunoprecipitates and the cell lysates were analyzed using SDS-PAGE and western blot, probing for EGFR and tubulin as loading controls. Shown are representative blots of three independent experiments (A). Densitometry analysis was performed to determine fold increase over control (B). (C-D) Polarized HEC-1-B (C) and T84 (D) cells were untreated or pre-treated with the EGFR kinase inhibitor AG1478 (10 nM) for 2 h then apically incubated with live GC with or without the inhibitor or gentamicin killed GC for 6 h. Cells were stained for β -catenin and GC and analyzed using confocal microscopy. The β -catenin FI of membrane compared to cytoplasm was determined as described in Fig. 4. Colocalization of GC microcolonies with β -catenin was analyzed for HEC-1-B (E) and T84 (F) polarized cells. Shown are the average ratios (\pm S.D.) from three independent experiments. ***, $p \leq 0.001$. **, $p \leq 0.01$.

suggests that GC-induced EGFR activation and its downstream signaling lead to the disassembly of the apical junction in GC-infected epithelial cells.

2.3.8 Negative regulation of the apical junction by GC facilitates GC transmigration

To investigate whether GC-induced redistribution of junctional proteins and reduction in the fence function of the apical junction contribute to GC infection, we utilized EGF and the EGFR kinase inhibitor to manipulate the effect of GC on the apical junction. Treatment with EGF, which activates EGFR signaling cascades and promotes junction disassembly [166, 167] resulted in a ~5-fold increase in GC transmigration across polarized HEC-1-B cells, as compared to the untreated control cells (Fig. 15A). In contrast, treatment with the EGFR kinase inhibitor, which inhibits the negative effects of GC on the apical junction, led to a ~5-fold decrease in GC transmigration compared to untreated control cells and a ~10 fold decrease when compared to EGF treated cells (Fig. 15A). T84 cells showed a similar increase and decrease in the presence of EGF and AG1478, respectively (Fig. 15B). These results suggest that GC-induced EGFR activation and the consequent disassembly of the apical junction facilitate the transmigration of GC across the epithelial monolayer.

2.3.9 Invasion of polarized cells is not influenced by EGFR activation

Previous research has determined that EGFR activation is involved in GC invasion into non-polarized cells [90]. Upon determining that transmigration across polarized cells is affected by EGFR activation, we wanted to determine if invasion was

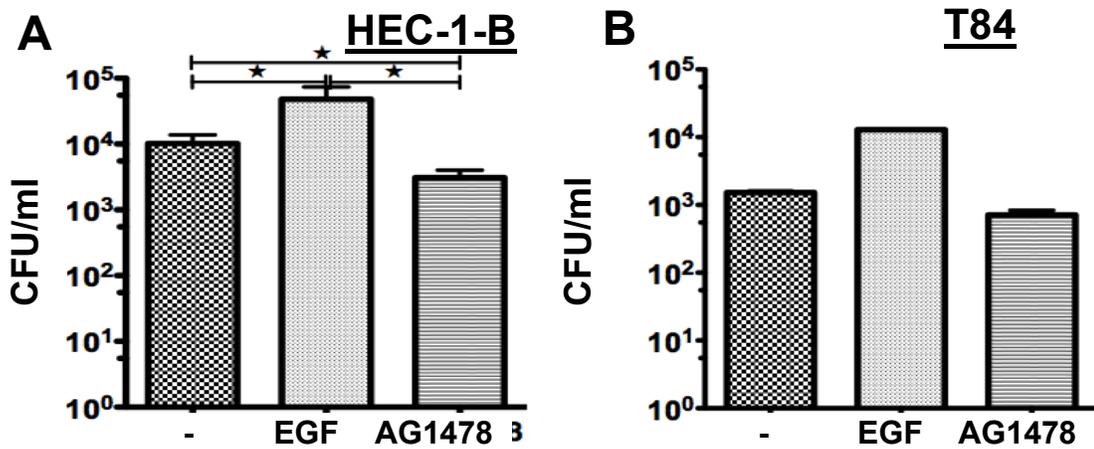


Figure. 15. GC transmigration is EGFR dependent. Polarized HEC-1-B (A) and T84 (B) cells were untreated or pre-treated with the EGFR kinase inhibitor AG1478 (10 nM) or EGF (10 nM) for 2 h and 1 h respectively, then apically incubated with GC for 6 h in the presence or absence of AG1478 or EGF. The basal media was collected and plated to determine transmigrated CFU. Shown are the average values (\pm S.D.) of more than three independent experiments for HEC-1-B and a single experiment for T84. *, $p \leq 0.05$.

also affected. Polarized HEC-1-B and T84 cells were pre-treated with EGF or AG1478 for 1 h and 2 h respectively, then apically inoculated with live GC at MOI 10 for 6 h. Cells were washed, exposed to gentamicin for 2 h, lysed and invaded CFU determined. Neither EGFR activation (EGF) nor inhibition (AG1478) caused any significant change in the number of GC that invaded cells, in either HEC-1-B (Fig. 16A) or T84 (Fig. 16B). While these results were surprising considering the observed results in non-polarized cells, we had already observed from other data, (Wang unpublished data) that polarized cells do not necessarily replicate phenomena observed in non-polarized cells. These results suggest that disruption of the junctional complex via EGFR activation leads to GC transmigration without affecting invasion of GC into these cells.

2.4 Discussion

GC establish infection in the female genital tract primarily by interacting with the endocervical epithelial monolayer. This monolayer of columnar epithelial cells is highly polarized and held together by the apical junction. This study provides the first detailed examination of the interaction of GC with polarized epithelial cells and reveals unique mechanisms underlying this interaction. Our results demonstrate that GC interaction with polarized epithelial cells induces the disassembly of the apical junction, weakening its barrier function against the lateral movement between the apical and basolateral membrane but not its barrier function against paracellular permeability. The negative effect of GC on the apical junction is dependent on GC-induced EGFR activation, which leads to the phosphorylation and disassociation of β -catenin from the apical junction. The

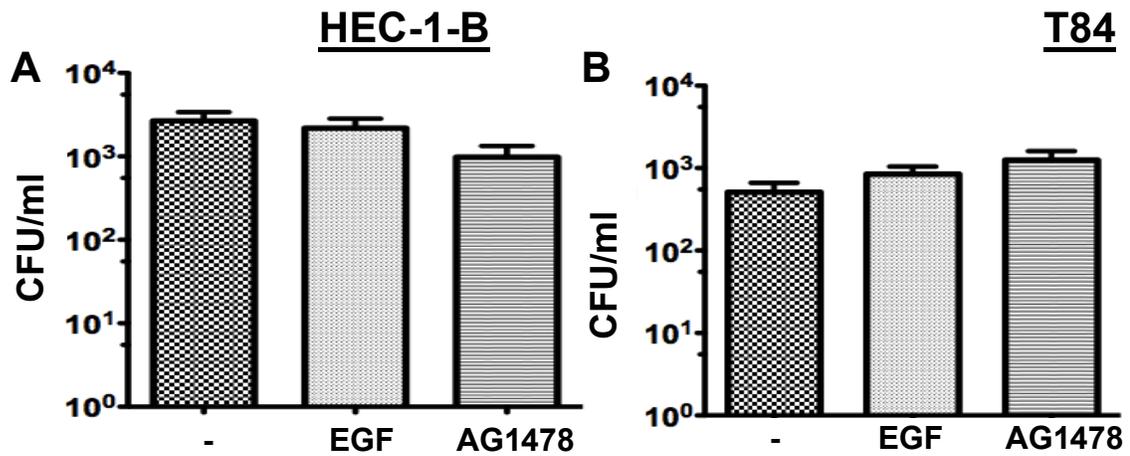


Figure. 16. GC invasion is unaffected by EGFR. Polarized HEC-1-B (A) and T84 (B) cells were untreated or pre-treated with the EGFR kinase inhibitor AG1478 (10 nM) or EGF (10 nM) for 2 h and 1 h respectively, then apically incubated with GC for 6 h in the presence or absence of AG1478 or EGF. Adherent bacteria were killed by gentamicin and invaded GC plated to determine CFU. Shown are the average values (\pm S.D.) from three independent experiments.

weakening of the apical junction by GC facilitates their transmigration across polarized epithelial cells, contributing to GC pathogenicity.

To establish infection, mucosal pathogens have to overcome our body's first line of defense, the epithelium. Therefore, it is not surprising that many mucosal pathogens, such as enteric bacterial pathogens enterohemorrhagic *E. coli*, *Salmonella* and *Helicobacter pylori*, are capable of disrupting the apical junction that secures the epithelial physical barrier, thereby increasing the permeability of epithelial monolayers lining the lumen of the intestine [46, 91, 160, 161, 168, 169]. Meningococci have been shown to disrupt both occludin based tight junctions and VE-cadherin-based intercellular junctions of endothelial cells, providing a mechanism for meningococci to cross the brain blood barrier [92, 93, 170]. GC have been shown to induce the redistribution of E-cadherin, but not the apical junctional proteins ZO-1 and occludin, in immortalized endometrial epithelial cells and isolated primary fallopian epithelial cells that are not polarized [171]. This study shows for the first time that the apical incubation of GC with polarized epithelial cells induces the protein redistribution of both adherens and tight junctions. Such redistribution was not only observed in low TER endometrial epithelial cells HEC-1-B, but also in colonic epithelial cells T84 that are highly polarized.

We examined how GC-induced redistribution of junctional proteins impacts the function of the apical junction. Different from the enteric bacterial pathogens and meningococcus, GC-induced redistribution of the junctional proteins does not lead to a significant increase in the permeability of epithelial monolayers (gate function). Instead, it weakens the lateral mobility barrier between the apical and basolateral membrane (fence function). This weakening allows proteins and lipids in the apical and basolateral

membrane to move more freely into each other, consequently reducing or losing the polarized functional domains in epithelial cells. How GC manage to affect the fence function of the apical junction more than its gate function is unclear.

Our findings that GC microcolonies preferentially localize at the cell-cell junction where junctional proteins such as β -catenin disassociate from support our hypothesis that the interaction of GC with multiple epithelia cells at their junction may prevent the expected free diffusion through paracellular space, even though GC promotes the disassembly of the apical junction. This occurs naturally when mucosal dendritic cells interact with polarized epithelial cells. The dendrites of the cells extend through the apical junction from the basolateral side to capture antigens at the apical surface, without increasing the permeability of the epithelium. This process is mediated by the direct interaction of junctional proteins expressed by dendritic cells with the apical junctional proteins of epithelial cells [172]. Whether the cell-cell junctional location of GC is mediated by direct interaction of GC with junctional proteins remains to be determined.

The dynamics of assembly and disassembly of the apical junction is tightly controlled by cell signaling [46, 71]. Many mucosal pathogens are capable of hijacking the host cell signaling apparatus to promote junction disassembly. For example, *H. pylori* injects CagA and other proteins via the type IV secretory apparatus into epithelial cells [173], where CagA targets to PAR1/MARK kinase complexes that play essential roles in epithelial cell polarity [174]. In the case of meningococcus, the bacteria disrupt the intercellular junction by activating β 2-adrenoceptor/ β -arrestin pathway in endothelial cells [175]. Here we show that GC induces EGFR activation in polarized epithelial cells, and that GC-induced redistribution of β -catenin and GC transmigration, but not invasion

across polarized epithelial cells, depends on EGFR activation. Our previously published data [90] demonstrate that GC trigger EGFR activation by inducing the expression and surface cleavage of EGFR ligands using a transactivation mechanism. Furthermore, EGFR is recruited from the basolateral surface to GC adherent sites at the apical surface, an additional indication of loss of apical-basolateral polarity [90]. EGFR activation is known to activate β -catenin by inducing its phosphorylation and release from the junctional complex. This leads to the disassembly of the apical junction and frees epithelial cells from cell-cell contact inhibitory mechanisms required for cell proliferation and migration [166].

The results from this study provide novel evidence for a link between GC-induced junction disassembly and GC transmigration across polarized epithelial cells. Our results show a significant increase in GC transmigration when the apical junction is disrupted by EGTA and a decrease in GC transmigration when GC-induced β -catenin dissociation from the apical junction is inhibited by the EGFR kinase inhibitor. However, the exact signaling mechanism that facilitates GC transmigration across polarized genital epithelial cells remains to be further examined.

There are two possible mechanisms for GC transmigration, an intracellular mechanism where GC invade epithelial cells from the apical surface, transcytose through the cells and exit from the basolateral membrane, and a paracellular mechanism where GC migrate through the apical junction. The cell-cell junction localization of GC, the positive correlation of GC transmigration with the junction disassembly and regulation of transmigration, but not invasion, suggest a possible paracellular pathway for GC transmigration. However, GC induced junction disassembly could facilitate GC

adherence and invasion as well. In polarized epithelial cells, the actin cytoskeleton is concentrated at the apical surface to support microvilli and the apical junction, thus strengthening the epithelial barrier. In contrast, some GC target molecules, such as CD46 [15] and EGFR [90], are mainly expressed on the basolateral surface [87, 176]. GC-induced reduction in the apical-basolateral polarity would allow these molecules to appear at the apical surface thus enhancing GC attachment and initiating signaling at the GC adherent sites. The disassociation of β -catenin from the apical junction can lead to detachment of the actin cytoskeleton from the apical junction and actin reorganization [52, 57, 149, 151], which can be utilized by GC for its invasion and transmigration. Since the EGFR kinase inhibitor does not completely block GC transmigration, it implies that there are additional mechanisms by which GC trigger transmigration.

Since EGFR is essential for the survival and polarization of epithelial cells, we were unable to use other approaches, such as si/shRNA knockdown, to confirm the results from the EGFR kinase inhibitor. Therefore, we utilized the approach of activating EGFR by addition of EGF. Our results show that EGF-induced EGFR activation increases GC transmigration, opposite of the effect of the EGFR kinase inhibitor that blocks EGFR activation and decreases GC transmigration, supporting the hypothesis that GC-induced EGFR transactivation is involved in GC transmigration.

Our results demonstrate that GC hijack the EGFR signaling pathway to breach the epithelial barrier for its transmigration. Further studies are required to define the cellular mechanisms by which GC transactivate EGFR and transmigrate across polarized epithelial cells. The resulting mechanistic knowledge will expand our understanding of GC pathogenesis and provide new ideas for preventive measures against GC infection.

Chapter 3: The Female Sex Hormones Estrogen and Progesterone Increase *Neisseria gonorrhoeae* Transmigration across a Polarized Epithelial Monolayer

3.1 Introduction

Gonorrhea, a sexually transmitted infection (STI) caused by *Neisseria gonorrhoeae*, manifests differently in men and women. The infection in women is often asymptomatic, thus leaving the infection untreated and unattended. This increases the risk of the development of the infection into more serious disease sequelae, such as pelvic inflammatory disease (PID) a major cause of infertility. However, in men the disease readily induces an inflammatory response allowing diagnosis followed by treatment [3, 6, 9, 177]. Due to anatomic differences of GC infection sites in men and women, the mechanisms used by GC to infect the male urethra versus the female reproductive tract may be different. In the male urethra, the bacteria establish an intimate interaction with the epithelial cells, inducing the secretion of cytokines and chemokines and the recruitment of leukocytes to the infection site. However, in infection of the lower female reproductive tract (FRT), GC interact with the epithelial cells causes membrane ruffling that facilitate bacterial internalization, but fails to elicit an immune response [13, 16, 29, 177]. Even though this significant difference in GC pathogenesis has been known for years, the mechanism underlying this difference has not been elucidated.

One of the major differences between the male urethra and female reproductive tracts is the female hormonal cycle that controls the menstrual cycle and prepares the FRT for pregnancy. Estrogen and progesterone are two of the major hormones of the

FRT, and their respective receptors can be cytoplasmic, nuclear or plasma membrane bound [101]. Binding of the hormones to the receptor leads to receptor activation and translocation to the nucleus if the receptor is cytoplasmic. The active receptor complex then binds to the DNA and activates transcription. This process is considered the ‘classical’ mechanism of hormone activation and requires 30-60 min to attain transcriptional activation. Hormone-receptor interaction can also induce fast (5-15 min) and transcriptional independent effects, which are considered ‘non-classical’ [101, 105, 178]. The non-classical effects appear to be induced by receptors on the plasma membrane, which activate several cytoplasmic signaling cascades including mitogen activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) [178]. Recent studies have shown that the non-classical receptor pathways and epidermal growth factor receptor (EGFR) signaling pathways are intertwined and synergize each other [107, 178], and manipulate the regulation and shedding of the endometrium [111].

GC research involving hormones focuses on effects in broth culture [179, 180]. These experiments show that progesterone in the $\mu\text{g/ml}$ range cause a concentration dependent decrease in GC growth. Where at 10 $\mu\text{g/ml}$, 70% of the control culture level remains after ~6 h incubation [179]. These levels are significantly higher than physiological peak progesterone serum levels of ~ 9 ng/ml [94] and of those used in this study (~15.7 ng/ml).

Many STIs are affected by the presence of sex hormones. Brabin *et al.* [114] reported that the stage of the menstrual cycle and use of oral contraceptives influence women’s susceptibility to sexually transmitted pathogens, such as chlamydia, Herpes

Simplex Virus (HSV), human immunodeficiency virus (HIV) and gonorrhoea. But the nature of the hormonal effects is dependent on the pathogen involved. The molecular and cellular mechanisms used by the hormones to either decrease or increase susceptibility are not fully elucidated. Using a mouse model of HSV, it has been shown that while estrogen has a protective effect against HSV, progesterone causes a significant increase in inflammation leading to death. Exposure to both estrogen and progesterone show increased protection against HSV. One mechanism proposed for the protective effect of estrogen is that estrogen induces thickening of the epithelial lining strengthening the epithelial barrier, but progesterone leads to thinning of the lining making it easier for the pathogen to cross the epithelium [181]. The hormones have a similar effect on Chlamydia infection, with the presence of estrogen abrogating the infection in mice while progesterone causes increased susceptibility [121]. Contrary to these results, inhibition of the estrogen receptor ($ER\alpha$ and $ER\beta$) caused a significant decrease in the infectivity of chlamydia in the endometrial epithelial cells HEC-1-B [182].

Similarly, clinical trials on the effects of contraceptive use on gonococcal infection generated inconsistent results. One study shows a protective effect of contraceptives [134] while others claim increased susceptibility [183-186], or no effect [132, 187-190]. Wu *et al.* examined the serum hormone levels of women with asymptomatic versus symptomatic GC infection and found a significantly higher level of progesterone in women with asymptomatic infection [133]. Replication of GC infection in the mouse model requires a long exposure to 17β -estradiol for a transient infection to occur [191]. The ability of this hormone to increase mouse susceptibility to GC infection [192] supports a role for 17β -estradiol in facilitating GC pathogenesis in the FRT. These

inconsistent observations exemplify the lack of a comprehensive and mechanistic understanding of the relationship between the female hormonal cycle and GC pathogenicity.

In this study we examine the effect of estrogen and progesterone on the various stages of GC pathogenesis in polarized cells. It is known that GC adhere to, invade into and transcytose across an epithelial layer facilitating pathogenesis [16, 17]. Most GC research has been performed on non-polarized cells, and the role of the female sex hormones in pathogenesis has not been examined. Our results show that while estrogen and progesterone have no effect on GC adherence and invasion into cells, they increase its transmigration across the monolayer.

3.2. Materials and Methods

3.2.1 Epithelial Cells

Human endometrial adenocarcinoma cell line, HEC-1-B cells (ATCC# HTB-113, Manassas VA, USA), were maintained in Eagles MEM, alpha medium supplemented with 10% heat inactivated fetal bovine serum (FBS). Cells were maintained at 37°C and 5% CO₂. Cells were seeded at 6×10^4 (6.5 mm diameter transwell) per transwell (3 µm pore size, polyester transwell inserts, Corning, Lowell, MA, USA) and cultured for ~10 days until transepithelial resistance (TER) reached ~400 Ω. TER was measured using a Millicell ERS volt-ohm meter (Millipore, Bedford, MA, USA).

3.3.2 *Neisseria Strains*

N. gonorrhoeae strain MS11 that expressed both pili and Opa (Pil⁺ Opa⁺) were used. Gonococci (GC) were grown on GC media base plates with 1% Kellogg's supplement (GCK) [154] for 15–18 h before inoculation. Pil⁺ Opa⁺ colonies were acquired based on their morphology using a dissecting light microscope. Bacteria were placed in suspension and the concentration determined using a spectrophotometer. GC were inoculated with epithelial cells at MOI 10:1.

3.3.3 *GC growth*

GC were grown in GC broth with Na₂CO₃ and Kelloggs supplement added. After an initial reading of 5, hourly readings were taken for 8 h, using the Klett machine that determines concentration based on the optical density of the solution at 640nm.

3.3.4 *Immunofluorescence analysis*

Cells were pre-treated with or without estrogen [5nM or 50nM] (Sigma-Aldrich, St. Louis, MO, USA) or progesterone [5nM or 50nM] (Sigma-Aldrich, St. Louis, MO, USA) for 60 h, and incubated with GC in the presence or absence of GC at MOI 10. Cell were washed and fixed using a pH shift method [155], permeabilized, and stained with anti-ZO-1 (BD Bioscience, Bedford, MA, USA) and anti-GC antibodies [156]. Cells were analyzed by confocal fluorescence microscopy (Zeiss LSM 510 or 710, Carl Zeiss Microscopy LLC, Thornwood, NY, USA). Z-series of images were obtained in 0.5 μm slices from the top to the bottom of cells, and three-dimensional (3D) composites obtained. The percentage GC clusters at the junctional complex and percentage of cells

showing discontinuous staining of junctional proteins was determined by visual inspection of multiple image slices.

3.3.5 Functional analyses of the apical junction

To determine the effect of GC on the gate function of polarized epithelial cells against the diffusion of molecules through the paracellular spaces, cells were incubated with GC and Lucifer yellow (50 μ M, Sigma, Saint Louis, MO, USA) or FITC (50 μ M, Acros Organics, Geel, Belgium) in the apical chamber for 6 h. The fluorescence intensity in the apical and basolateral media was then determined using a fluorometer.

3.3.6 GC adherence, invasion and transmigration assays

Polarized epithelia cells were untreated or pre-treated with ICI 182780 [5 μ M] (ER inhibitor) or mifepristone [5 μ M] (PR inhibitor) and estrogen or progesterone, then incubated apically with GC for 3 h (adherence) or 6 h (invasion and transmigration) at 37 °C. Cells were washed, lysed and plated on GCK after 3 h incubation to determine adherent CFU. Media from the basal compartment was collected and plated onto GCK to determine the number of transmigrated bacteria. Or after incubation cells were exposed to gentamicin (100 μ g/ml) for 2 h, washed, lysed and plated to determine invaded bacteria.

3.3 Results

3.3.1 Sex Hormones do not affect GC growth

The sex hormones, estrogen and progesterone, are a significant component of the female reproductive system. The levels of these hormones vary throughout the menstrual

cycle and control the shedding and regeneration of the epithelial lining of the FRT. Thus their role in GC infection in the reproductive system must be taken into account when trying to decipher mechanisms underlying GC pathogenesis. Previous work has shown that progesterone causes a decrease in GC growth in a time and concentration dependent manner [179, 180], but there was no analysis of the effect of 17 β -estradiol on GC growth. To determine if either estrogen or progesterone affected GC growth, MS11 P⁺O⁺ GC collected from a 12 h GC plate were cultured in GC growth media in the absence of hormones or the presence of 17 β -estradiol (50nM) or progesterone (50nM). Density readings were taken hourly and plotted (Fig. 17). These results show that the presence of neither 17 β -estradiol nor progesterone causes any change in the growth pattern of GC at the concentration utilized for these experiments.

3.3.2 Hormones slightly increase the localization of GC clusters at the cell-cell junction

We have previously shown that live, but not gentamicin killed GC, preferentially localize at the cell-cell junction of polarized epithelial cells [Edwards 2012 submitted]. To investigate if sex hormones have any effect on GC localization, polarized HEC-1-B cells were pretreated apically and basally with estrogen or progesterone for 60 h then apically inoculated with GC at MOI 10 for 6 h in the absence or presence of hormones. Cells were fixed, permeabilized and stained for the ZO-1 and GC using specific antibodies, then analyzed using confocal microscopy. GC clusters at the cell-cell junction were visually identified and counted based on their spatial location to the junctional protein ZO-1 (Fig. 18A). The data shows that in the absence of hormones approximately 85% of GC clusters localized at the cell-cell junction, and in the presence of both

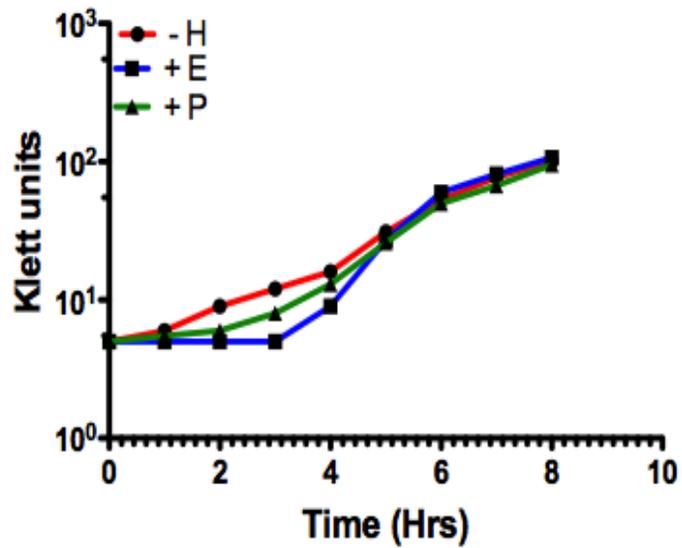


Figure 17. Sex hormones do not affect GC growth.

MS11 P⁺O⁺ was grown in GC broth. GC at an initial Klett OD of 5 was added to the growth media and Klett turbidity readings taken hourly for 8 h.

estrogen and progesterone GC at the cell-cell junction increased to 93% and 95%, respectively. These results indicate that hormones increase the localization of live GC clusters at the cell-cell junction.

3.3.3 Hormones enhance GC induced redistribution of the junctional protein ZO-1.

The increase in the percentage of cell-cell junctional localization of GC clusters indicates a possible role for hormones in facilitating GC-induced junction disassembly. To test this hypothesis, polarized HEC-1-B cells were pre-exposed to hormones then incubated with GC for 6 h in the continued presence of hormones. The apical junction was stained for ZO-1 and fluorescence confocal microscopy used to analyze the cellular distribution of ZO-1. In the absence of GC and hormones, ZO-1 staining appeared continuous along the cell membrane close to the apical surface (Fig. 18Ba), confirming polarization of HEC-1-B cells. Hormone treatment alone did not significantly change the distribution pattern of ZO-1 (Fig. 18B d, Bg). However, in the presence of GC, cells lost the continuous peripheral staining pattern of ZO-1, suggesting that there is disruption of the apical junction. We determined the percentage of epithelial cells with discontinuous ZO-1 staining as a quantitative measure for junctional disruption (Fig. 18B b,c,e,f,h,i). GC inoculation alone increases the percentage of epithelial cells with discontinuous staining from 16% to 74% (Fig. 18B). Hormone treatment with either 17β -estradiol or progesterone did not significantly increase this percentage. However, when comparing the distribution pattern, it was noted that ZO-1 staining in hormone treated and GC infected cells (Fig. 18Bd,e,h,i) appeared more disorganized than cells not exposed to hormones (Fig. 18Bb,c). These results suggest that while the hormones do not

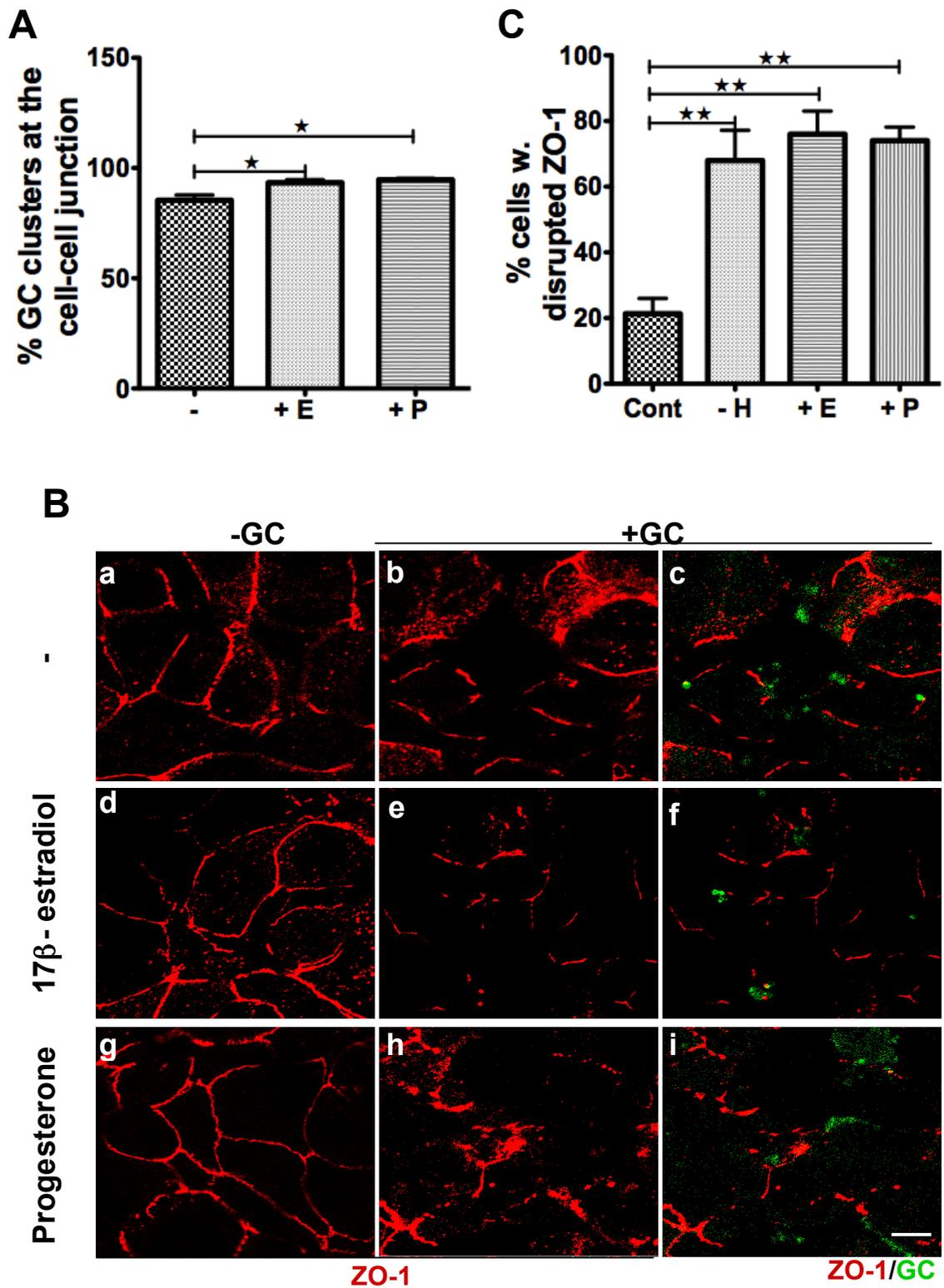


Figure 18. Hormones slightly increase the localization of live GC at the cell-cell junction, and enhance ZO-1 re-distribution in the presence of GC .

Polarized HEC-1-B cells were unexposed (Ba-c) or pre exposed to estrogen (E) (Bd-f) or progesterone (P) (Bg-I) for 60 h and then apically inoculated with live GC (P⁺O⁺ MS11) (Bb,c, Be,f, Bh,I) at MOI 10 6 h. Cells were fixed, permeabilized and stained for ZO-1 and GC, and analyzed using confocal microscopy. The number of GC clusters localized at the cell-cell junction marked by ZO-1 was quantified by visual inspection (A). The number of host cells with disrupted peripheral ZO-1 staining was also visually analyzed and the average percentages (\pm S.D.) from three independent experiments were reported (C). Scale 10 μ m. *, p \leq 0.05. **, p \leq 0.01.

increase the number of epithelial cells with disrupted junctional complexes, they enhance the effect of junctional disruption.

3.3.4 Permeability of the Junctional Complex is not affected by the presence of hormones

Since junctional disruption was observed we needed to determine if the functionality of the junction was also affected by the presence of hormones in synergy with GC. Polarized HEC-1-B cells were unexposed or exposed to 17β -estradiol or progesterone and then apically inoculated with GC and Lucifer yellow dye (500 μ M) for 6 h. The basal and apical media were collected and the amount of Lucifer yellow was determined using a fluorometer. The Fluorescence Intensity Ratio (FIR) of Lucifer yellow in the basal to apical medium was calculated as an index of permeability. We did not detect any change in the permeability of the cells in the presence of 17β -estradiol or progesterone without (data not shown) or with GC inoculation (Fig. 19). These data suggest that while GC and hormones have a synergistic effect on the redistribution of the junctional protein ZO-1, they do not increase the permeability of the epithelium.

3.3.5 Treatment with hormones increases GC transmigration without affecting GC adherence and invasion

To determine whether the hormonal cycle of the FRT has any impact on GC infection, we examined the effects of 17β -estradiol or progesterone treatment on CG adherence to, invasion into, and transmigration across polarized epithelial cells: all critical events for GC to establish infection. Polarized HEC-1-B cells were pre-treated

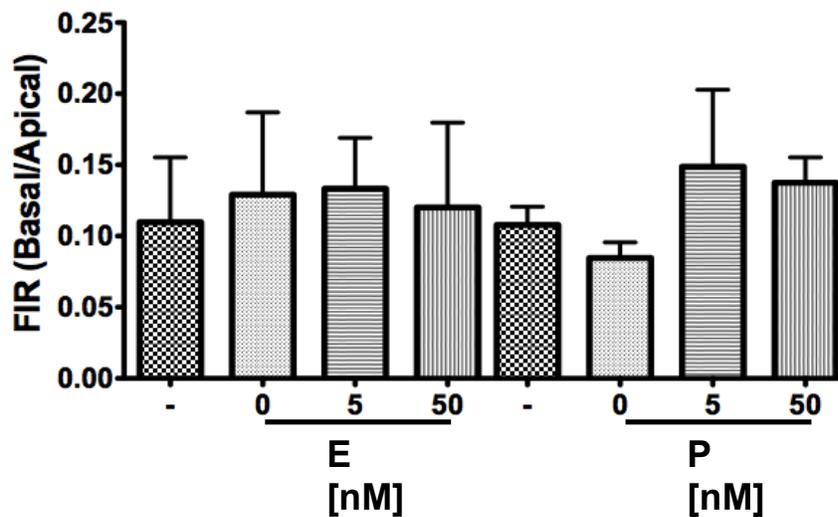


Figure 19. Permeability of the junctional complex is not affected by the presence of hormones. Polarized HEC-1-B cells were unexposed to various concentration of estrogen (E) or progesterone (P) for 60 h and incubated with or without GC for 6 h in the presence of Lucifer yellow dye. The fluorescence intensity of Lucifer yellow in the apical and basal compartments was determined using a fluorometer. The basal compared to apical FIR of Lucifer yellow was determined. Shown are the averages values (\pm S.D.) from three independent experiments.

with 17β -estradiol or progesterone then apically inoculated with GC for 3 h in the continued presence of hormones. Cells were washed to remove non-adherent bacteria, lysed and plated to determine epithelial cell associated GC. We found that the presence of neither 17β -estradiol nor progesterone cause a change in the level of GC adherence compared to cells that were not treated with hormones (Fig. 20A). To determine the number of invaded GC, incubation was extended to 6 h. The extracellular bacteria were killed by incubation with gentamicin and gentamicin resistant GC were determined as invaded bacteria. The results show that neither a low (5nM) nor high (50nM) level of 17β -estradiol or progesterone (Fig. 20B) causes any change in GC invasion.

To determine the effect of hormones on GC transmigration, polarized HEC-1-B cells were exposed to hormones and apically inoculated with GC for 6 h in the presence of the hormone. The number of GC in the basal medium was determined as transmigrated GC. Both 17β -estradiol (Fig. 20C) and progesterone (Fig. 20D) at the 50 nM concentration caused an ~ 5 fold increase in GC transmigration over hormone free cells. There was no significant increase observed at the lower hormone concentration. EGTA treatment, which completely disrupts the apical junction, showed ~ 25 fold increase in GC transmigration compared to untreated epithelial cells (Fig. 20C, D).

These results collectively show that the sex hormones estrogen and progesterone, which synergize with GC in disrupting the apical junction of polarized epithelial cells, increase GC transmigration without affecting GC adherence and invasion.

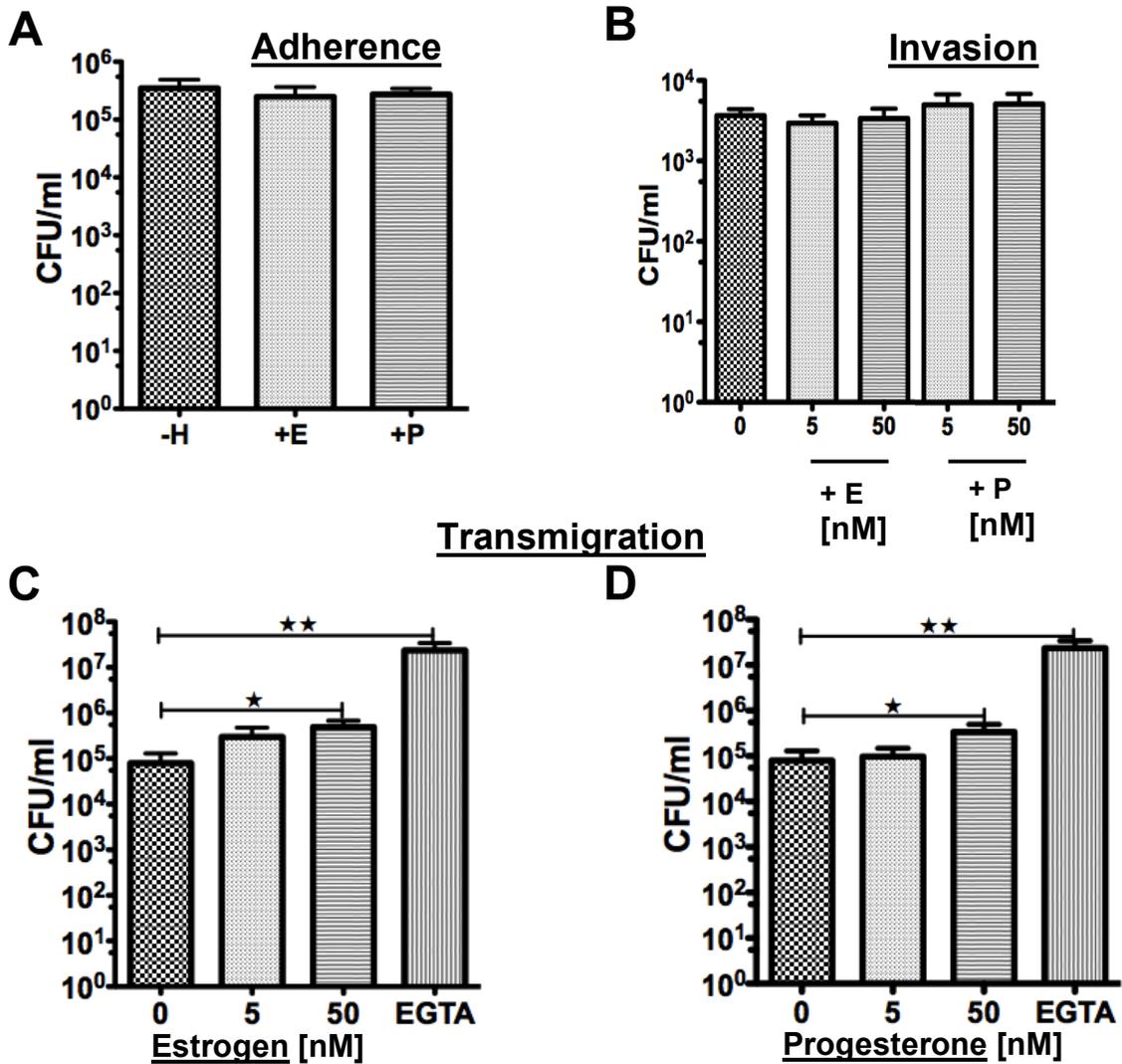


Figure 20. Transmigration, but not adherence or invasion is affected by 17 β -estradiol or progesterone. Polarized HEC-1-B cells were unexposed or pre exposed to estrogen (E) or progesterone (P) for 60 h and then apically inoculated with live GC (P⁺O⁺ MS11) at MOI 10 for 3 h or 6 h. After 3 h cells were lysed and adherent bacteria plated on GCK (A). After 6 h GC inoculation cells were exposed to gentamicin for 2 h then lysed to determine invaded bacteria (B) or the basal media plated to determine transmigrated CG (C,D). Shown are the average CFU (\pm S.D.) from three independent experiments. *, $p \leq 0.05$. **, $p \leq 0.01$

3.3.6 Inhibition of the classical hormone receptors does not reverse the effect of 17 β -estradiol nor progesterone on GC transmigration

To investigate if the hormones increase GC transmigration by activation of their receptors, we inhibited these receptors with classical receptor inhibitors ICI 182780 (17 β -estradiol) or mifepristone (progesterone). ICI 182780, is a pure antiestrogen that completely blocks the activity of the ER by binding to the ER and causing increased degradation and reduced dimerization of the receptor [103]. When mifepristone binds to the PR, it interacts with amino acids within the binding region and this induces a conformational change within the ligand binding domain that prevents interaction of PR with its cellular targets [104]. The classical receptor inhibitors inhibit the cytoplasmic and nuclear hormone receptors, but are less effective on the subset of receptors that reside on the plasma membrane and interact with GPCR and EGFR [178, 193]. There are no effective inhibitors available that directly inhibit the non-classical hormone receptors. To investigate if the inhibition of the classical receptors would block the effect of the hormones on GC transmigration, polarized HEC-1-B cells were pre-incubated with ICI 182780 or mifepristone at 5 μ M for 90 h before GC inoculation. At 60 h before inoculation, cells untreated or treated with inhibitors were incubated with 17 β -estradiol or progesterone. Cells were apically inoculated with GC for 6 h, and transmigrated bacteria in the basal media were enumerated. The data show no significant difference in GC transmigration between cells that were treated with or without the hormone receptor inhibitor (Fig. 21). This result suggests that the classical hormone receptors may not be involved in facilitating GC transmigration.

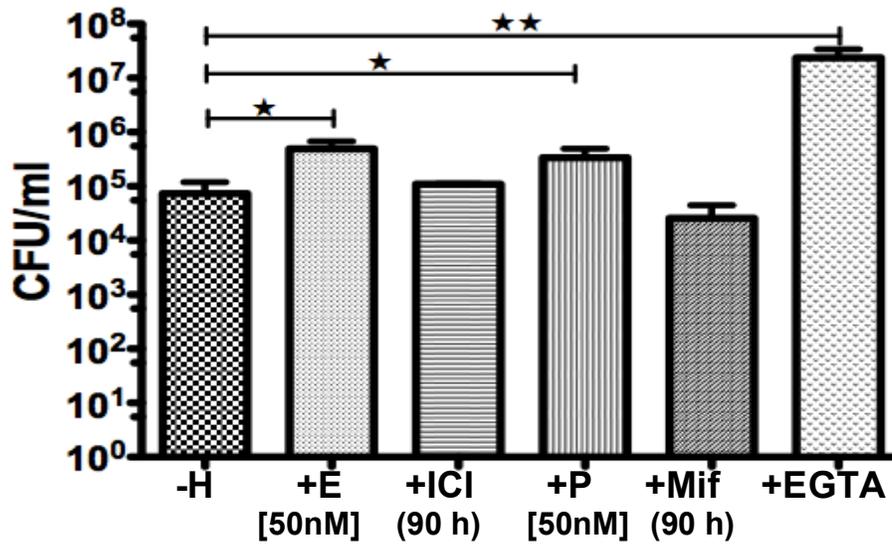


Figure 21. Neither 17 β -estradiol nor progesterone effects on transmigration are reversed by the presence of classical hormone receptor inhibitors.

Polarized HEC-1-B cells were unexposed to hormones or pre treated with the receptor inhibitors to estrogen (ICI) or progesterone (Mif) for 90h and/or exposed to estrogen or progesterone for 60 h then exposed to GC for 6 h. Some cells were apically and basally exposed to EGTA [5nM] for 10 min then washed and exposed to GC for 6 h. Basal media was collected and plated to determine transmigrated GC. Shown are the average CFU (\pm S.D.) from two independent experiments.

*, $p \leq 0.05$. **, $p \leq 0.01$.

3.3.7 Inhibition of EGFR in the presence of 17 β -estradiol or progesterone affects GC transmigration

Since the classical hormone receptor pathway does not appear to be involved in GC transmigration, we decided to determine if the non-classical pathway might be involved. There are exclusive inhibitors to the non-classical pathway, but since there appears to be crosstalk between the receptors and EGFR [105,173], we decided to inhibit EGFR kinase activity in the presence of the hormones and determine if this affected GC transmigration. Polarized HEC-1-B cells were incubated with 17 β -estradiol or progesterone for 60 h. Cells were then exposed to AG1478 [10nM] for 2 h pre then apically inoculated with GC for 6 h, and transmigrated bacteria in the basal media were enumerated.

The results show that while the presence of the hormones increase GC transmigration, addition of the EGFR kinase inhibitor reduces transmigration under all conditions to a similar level (Fig. 22). Thus essentially transmigration in the presence of the hormones and AG1478 is reduced by a greater degree. This suggests that EGFR may be synergistically activated by the sex hormones and GC.

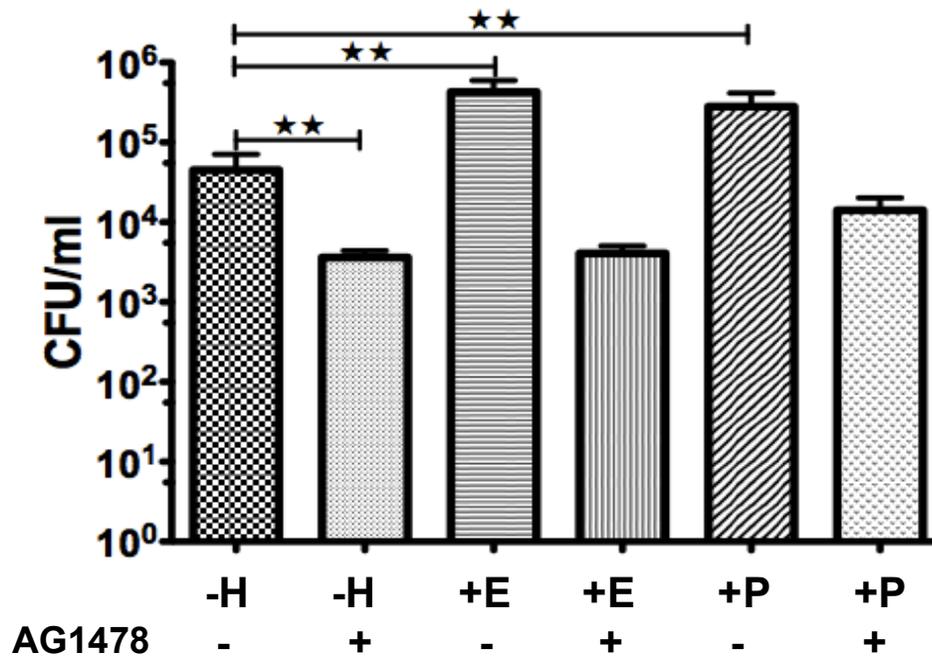


Figure 22. Inhibition of EGFR kinase activity reduces GC transmigration in the presence of both 17 β -estradiol and progesterone. Polarized HEC-1-B cells were unexposed or treated with hormones for 60 h, then some cells were exposed to AG1478 for 2 h before inoculation with GC for 6 h. Basal media was collected and transmigrated bacteria plated on GCK plates. Shown are the CFU (\pm S.D.) from a experiment. **, $p \leq 0.01$.

3.4 Discussion

The female sex hormones 17 β -estradiol and progesterone play a significant role in determining the environment of the female reproductive tract (FRT). During the menstrual cycle estradiol levels peak at day 12 (~0.2 ng/ml) and progesterone peaks at day 23 (9 ng/ml). As the hormones fluctuate during the various stages of the menstrual cycle, they modulate the FRT and local immunity to accommodate reproductive changes and prepare for pregnancy [194, 195]. This study shows that physiological levels of both of these hormones enhance GC-induced disruption of the apical junction complex and cause an increase in GC transmigration. This suggests that the rise in estrogen and progesterone levels during the menstrual cycle potentially increase the susceptibility of women to GC infection and provide GC with two time windows of opportunity.

Previous studies by Fitzgerald and Morse showed an inhibitory effect of progesterone on GC growth when GC were cultured with the hormone [179, 180], however, the GC strains studied did not include the MS11 strain and the concentrations analyzed were much higher than physiological levels and over 600-fold greater than that being utilized for these experiments. We found that neither progesterone nor estrogen caused a change in GC growth. The concentrations of progesterone (15.7 ng/ml) and estrogen (13.6 ng/ml) used in my studies were approximately 2-fold and 17-fold higher than blood levels respectively. While the exact hormone concentrations at the FRT are not known, they are generally higher than blood levels since they are locally secreted.

Our research has shown that viable GC not only localize to the apical junctional complex, but also cause disruption of numerous junctional proteins, leading to reduced functionality of the junction and GC transmigration. The female sex hormones slightly

increase localization of GC clusters at the apical junction but do not significantly change the redistribution of ZO-1 from the periphery of the polarized cells. Since we hypothesize that GC clusters localizing at the cell-cell junction assist in facilitating pathogenesis, this increased localization of clusters at the junction in the presence of hormones would imply increased GC transmigration when hormones are present.

Many pathogens overcome our body's first line of defense, the epithelium by disrupting the apical junction. These pathogens, which include *Enterohemorrhagic E. coli*, *Salmonella* and *Helicobacter pylori*, destroy the junction thereby increasing the permeability of epithelial monolayers [46, 91, 160, 161, 168, 169]. Some pathogens such as *Toxoplasma gondii* can paracellularly cross the monolayer without destroying the apical junction [91], a phenomenon that GC might also be capable of performing. The other *Neisseria* pathogen, *meningitidis*, disrupts both occludin based tight junctions and VE-cadherin-based intercellular junctions of endothelial cells, providing a mechanism for crossing the brain blood barrier [92, 93, 170]. Studies in epithelial cells show that the bacteria are capable of transmigrating across the polarized epithelia without disrupting the junctional complex [196, 197]. If the junction is not destroyed but only transiently disrupted, permeability to small molecules might not be observed. Since the GC clusters localize on the junction they might fill the space thus preventing the molecules from going through, or might interact with the junction as they themselves paracellularly cross. Either mechanism would prevent small molecules, such as dyes from freely moving from the apical to basal compartment as observed with the Lucifer yellow dye.

Adherence and invasion, two early stages of pathogenesis, were not affected by the presence of either 17β -estradiol or progesterone. However, there was increased GC

transmigration observed in a concentration dependent matter within the physiological range. Estrogen at 5nM and 50nM caused approximately a 5 and 10-fold increase respectively, over the no hormone control. At 5nM progesterone did not cause an increase over the no hormone control, but there was a 5 fold increase in the presence of 50nM of progesterone. One mechanism of GC transcytosis could be the intracellular pathway, in which GC are apical endocytosed, traverse to the basolateral membrane and are exocytosed from the basal surface. Thus an increase in transcytosis should be concomitant with an increase in invasion. The fact that we observed no invasion increase, but a transmigration increase suggests that GC might be utilizing the paracellular pathway. This mechanism allows for the passage of GC between cells from the apical to basal compartment. The increased degree of junctional disruption in the presence of the hormones strengthens this possibility. Additionally, chemical destruction of the junctional complex by EGTA had no effect on GC invasion but facilitated a significant increase in transmigration, further reinforcing the paracellular pathway as a potential mechanism for GC transmigration.

This study finds that the increase in GC transmigration by hormone treatment was not blocked by inhibition of the classical hormone receptors. While the efficacy of the inhibitors on hormone receptors needs to be confirmed, this result suggests that the mechanism involved in this transmigration event may not be mediated via the classical hormone activation pathway. This finding indicates a dependency of GC transmigration on the activation or activity of non-classical hormone receptors. Plasma membrane localized, non-classical receptors are believed to be more associated with signaling events and are generally not significantly inhibited by classical inhibitors [101, 105, 178, 193].

Since these receptors also cross talk with EGFR, activation of these receptors by GC may explain the phenomenon observed. Inhibition of EGFR in the presence of the hormones caused a decrease in GC transmigration that was greater than the decrease observed when hormones were not present.

The mechanism utilized for hormone-induced increase in transmigration is unknown. Either the hormones are activating the plasma bound receptors involved in non-classical hormone activation [101, 105, 178, 193] or GC itself might be interacting with these hormone receptors. Non-classical hormone receptors activate a number of signaling cascades [101] and synergize with EGFR signaling. Our previous studies demonstrated that GC can induce the transactivation of EGFR in epithelial cells and that EGFR activation is required for invasion into non-polarized cells and transmigration across polarized cells [90]. These findings suggest that GC and the hormones might be working in synergy to activate the EGFR signaling pathway to disrupt the apical junction and facilitate GC transmigration. Further experiments would have to be performed to determine the cellular mechanism(s) utilized for GC transmigration.

In summary, our findings suggest that 17- β estradiol and progesterone play a role in GC pathogenesis. However, these findings only open the door to understanding the role of these sex hormones in the progression of gonococcal infection. Such knowledge will provide new strategies for preventative and therapeutic measures for GC infections in women.

Chapter 4: Conclusions

4.1 General Summary

Gonorrhea is the second most commonly reported sexually transmitted infection, and is caused by the human obligate gram-negative bacterial pathogen, *Neisseria gonorrhoeae* (GC). While the infection is typically recognized and cleared by the immune system in males, the infection in females often remains asymptomatic and thus untreated. Because it remains untreated it can progress into more complicated forms, such as pelvic inflammatory disease (PID). PID can lead to scarring of the fallopian tubes and cause other serious complications within the female reproductive system including ectopic pregnancies and infertility [3, 6, 7]. In addition to these reproductive complications, it has been observed that GC infection correlates with increased HIV infectivity [135, 136]. To progress to these complicated forms, the pathogen crosses the protective epithelial monolayer of the female reproductive tract (FRT) and invades the underlying tissues. Invasion into the underlying tissues also allows for evasion of the immune system, since once the bacteria are within the epithelial cells they are not exposed to cells of the immune system [13, 16]. The monolayer consists of apical junctional complexes that tightly hold neighboring cells together and prevent paracellular movement between cells. The junction also ensures that proteins and lipids remain in their appropriate apical or basolateral membrane location, maintaining functional polarization of the two surfaces [28, 35, 47].

Many bacterial and viral pathogens have developed mechanisms to disrupt the junctional complex to facilitate their infection. The majority of gonococcal research has been focused on non-polarized cells, thus how the interaction of GC with polarized

epithelial cells impacts the apical junction and GC pathogenesis has not been well studied. Based on the data from GC infection generally in non-polarized epithelial cells *in vitro*, GC infection has been proposed to occur through the stages of attachment, adherence, invasion, intracellular survival and exocytosis. Attachment and adherence are the early stages of infection and occur within the first few hours following exposure, this is followed by invasion into the cell within 4-6 hours. The bacteria survive within vacuoles or directly in the cytoplasm and can be found exocytosed approximately 18-40 h after inoculation, dependent on the GC strain used. The surface molecules pili, opa and LOS are all considered necessary for infectivity and pathogenicity.

The goals of this study were two-fold, (1) to determine if the interaction of GC with polarized epithelial cells affect the apical junction and if EGFR, which is known to be transactivated by GC in non-polarized epithelial cells, is involved in this process, and (2) to determine if the female sex hormones, estrogen and progesterone, play any role in GC pathogenesis. I developed these two projects independent of each other in order to explore two important aspects of GC pathogenesis that have not previously been examined. My studies show that when GC interact with polarized epithelial cells they prefer to localize around the apical junction. The junctional location of GC is concurrent with disassociation of the TJ proteins ZO-1 and occludin from the junction. Their disassociation leads to the disruption of the TJ and the association of the TJ with the actin cytoskeleton. In addition to the TJ proteins, GC inoculation also induced the redistribution of an adherens junction protein, β -catenin from the plasma membrane to the cytoplasm. This disassembly of the apical junction reduces the 'fence' function of the junction, which keeps surface proteins and lipids in their appropriate apical or basolateral

location, but does not significantly affect its 'gate' function, which controls the movement of molecules via the paracellular space.

My studies show that degradation of the junctional proteins is not the mechanism for GC-induced junction disruption, since GC inoculation does not change the cellular levels of these junctional proteins. Instead, GC inoculation induces the tyrosine (Tyr) phosphorylation of β -catenin, which is known to lead to its redistribution away from the cell-cell junction [45, 48, 52, 54, 56, 58]. The presence of GC also leads to increased EGFR phosphorylation, a phenomenon previously observed by Swanson *et al* [90] in non-polarized cells. Using the EGFR kinase inhibitor, AG1478, I demonstrate that the dissociation of β -catenin is dependent on EGFR kinase activity. My results further show that disrupting the apical junction by EGTA increases GC transmigration and blocking GC induced junctional disruption by EGFR inhibitors reduces GC transmigration. These data collectively demonstrate that GC induced transactivation of EGFR triggers the phosphorylation of β -catenin [79], which leads to the disassembly of the apical junction and facilitate GC transmigration.

Previous transmigration analysis within the field has shown transmigration to be observed approximately 24 h post inoculation [4, 33-35]. During the course of my project I have consistently observed transmigration at the 6 h time point. This difference may be the result of the GC strain used and/or the stage of the infection process that was analyzed. My experiments used the MS11 strain, while the previous experiments used the FA1090 strain. While both originated as clinical isolates, FA1090 lacks a portion of the GC genome that is found in all other pathogenic strains [198]. Transmigration may occur in either of two manners, intracellular or paracellular. It is possible that the time points

analyzed in my experiments as compared to other experiments may be analyzing different stages of the infection process and possibly different forms of transmigration. The observed disruption of the junctional complex proteins indicates that paracellular transmigration is a feasible process that has not previously been extensively analyzed within the GC field.

The second goal of this thesis examines the role that estrogen and progesterone play in GC pathogenesis. Female sex hormones control the tissue reorganization of the female reproductive tract through the menstrual cycle and present a major difference between GC infection sites in male and female [94, 96, 177]. However, the relationship between the hormones and GC pathogenesis in the FRT has not been well examined. The effect of oral contraceptives and by extension hormones on susceptibility to GC infection is controversial and inconclusive. This makes a detailed investigation into the role of estrogen and progesterone even more relevant.

My research shows that physiological levels of estrogen and progesterone have no effect on the growth of GC. While they do not affect the percentage of host cells with disrupted tight junctions, they increase the percentage of GC localized at the cell-cell junction and the extent of junctional disruption. Unfortunately, there is currently no efficient method to quantify the extent of junction disruption. Neither hormone causes an increase in junctional paracellular permeability in the absence or presence of GC, leaving the 'fence' function unchanged. While physiological relevant levels of estrogen or progesterone have no effect on GC adherence and invasion, they increase GC transmigration in a concentration dependent manner. However, the classical hormone receptor inhibitors ICI 162,780 (ER) and mifepristone (PR) do not block hormone-

enhanced GC transmigration, suggesting the hormones exert their effects via a non-classic pathway(s). Inhibition of EGFR kinase activity causes a greater decrease in GC transmigration, than that observed in the absence of hormones. These results support the role of the non-classical hormone receptor pathway in GC transmigration, since this pathway has been shown to work in synergy with the EGFR pathway.

Taken together these results provide new insights into GC pathogenesis in the FRT where the female sex hormones are present. Our data suggest that the hormones synergize with GC to induce junctional disruption, consequently enhancing GC transmigration. GC breach the apical junction via transactivation of EGFR. EGFR is not only transactivated by GC [90], but has also been shown to be activated by ‘non-classical’ hormone signaling [101, 105, 107]. I postulate that there is a synergistic relationship between the hormones and GC in the activation of EGFR receptors, which leads to the enhanced junction disruption and the increase in GC transmigration. Our findings that inhibition of EGFR activity reduced, while EGF increased, transmigration advocates a role for EGFR in GC transmigration supporting this hypothesis. I propose a working model utilizing a synergistic functional interaction between GC and the hormones to explain GC transmigration in the context of the sex hormones (Fig. 23).

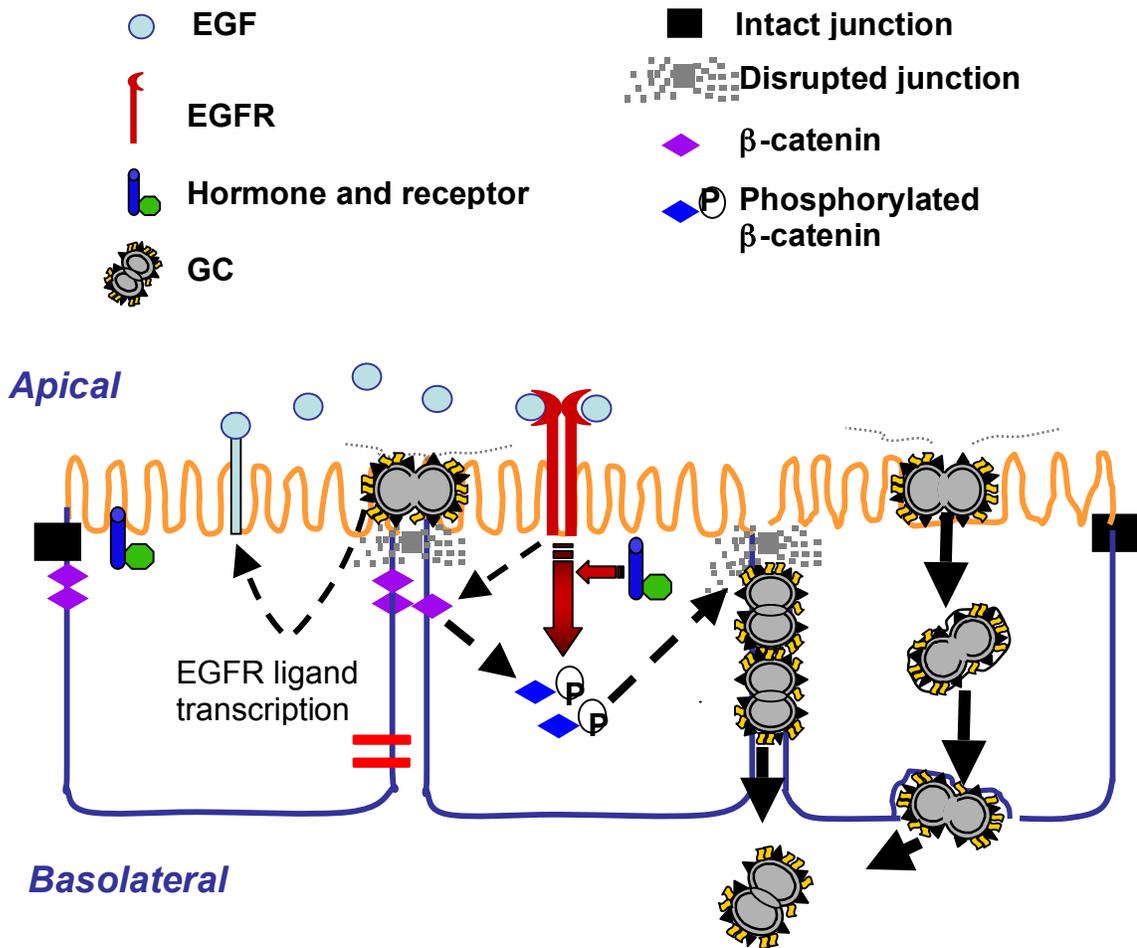


Figure 23. Working model of EGFR activation leading to increased GC transmigration and the proposed synergistic effect of hormones on this process.

GC interact with host cells, transactivate EGFR leading to the phosphorylation of β -catenin and its disassociation from the junction, thus reducing the fence function.

More EGFRs become apically localized and GC transmigrates across the monolayer.

The presence of hormones facilitates activation of EGFR by an unknown mechanism, so when GC associate with the cells the junctional complex is already weakened. This

leads to increased transmigration of the bacteria across the monolayer.

Hormones interact with their receptors or other as yet unknown receptors, leading to activation of EGFR signaling cascades by a currently unknown mechanism(s). This hormone-mediated EGFR activation synergizes with GC-induced EGFR transactivation. EGFR activation causes phosphorylation of β -catenin and its disassociation from the apical junction. Disruption of the junctional complex causes a decrease in the fence function of the apical junction, which allows basolaterally located EGFR to laterally move to the apical surface, so GC have an increased number of receptors to transactivate. The disruption of the junctional complex is perpetuated and GC transmigration increased. In the absence of hormones GC is able to transactivate EGFR leading to junctional disruption and reduced fence function, but without activation of EGFR by the sex hormone receptors there is overall reduced EGFR signaling. The actual mechanism that leads from EGFR transactivation to β -catenin phosphorylation and junction disruption needs to be elucidated.

There are two other mechanisms that potentially contribute to this process. First, estrogen and progesterone interact with and activate host surface receptors other than EGFR, such as trimeric G-protein coupled receptors, and use activated signaling cascades to disrupt the apical junction and allow GC targeted host receptors to be available on the apical surface. Second, GC may directly or indirectly interact with the hormone receptors and activate them, facilitating the junction disruption and increasing GC transmigration. Additional research, especially with regards to signaling needs to be undertaken.

4.2 Future Directions

The results obtained from this study and the resultant implications with regards to gonococcal pathogenesis open a window into the mechanism(s) used to facilitate GC transmigration. Further studies are required to fully elucidate the pathway from EGFR to β -catenin phosphorylation as well as the actual mechanism involved in the hormone based increase in GC transmigration.

GC causes EGFR transactivation [90], but the resultant downstream pathway that leads to β -catenin phosphorylation is unknown. While Erk is believed to be involved in EGF induced phosphorylation of β -catenin [150] it is not known if this is the pathway activated by GC. Inhibition studies and the resultant effect on GC transmigration must be performed. Immediate EGFR targets including phospholipase C gamma (PLC γ), phosphatidylinositol 3 kinase (PI3K) and Ras as well as proteins further downstream such as protein kinase C (PKC), Akt and Erk can be analyzed. Knockdown siRNA studies and/or inhibitory chemicals or proteins can be used to perform these experiments.

Phosphorylation of β -catenin at Tyr 654 is important for its binding to E-cadherin and thus its localization at the cell-cell junction [58]. This Tyr residue can be mutated to Phe and the effect on its redistribution to the cytoplasm and the resultant effect on GC transmigration can be observed.

A functional AJ is required for the formation of the TJ, making the AJ a crucial component of the junctional complex. While I analyzed the effect of β -catenin phosphorylation on its localization and inferred the effect that this would have on the junction, a more detailed analysis confirming reduced junctional functionality should be performed. Additionally, the effect on the phosphorylation levels of the other junctional

proteins in the absence or presence of GC should be analyzed. Any role that EGFR plays in this process should also be elucidated.

While my study shows that the presence of estrogen and progesterone leads to increased GC transmigration, the actual mechanism underlying this phenomenon has not been examined. In order to examine if a synergistic relationship exists between GC and the sex hormones, signaling cascades must be examined. We know that GC activate EGFR [90] and both estrogen and progesterone can activate Erk 1/2, downstream components of EGFR pathway [107]. Gentamicin killed GC can be used instead of viable GC to observe any changes in signaling. Combinations of killed GC with hormones or viable GC with inactive hormone homologs can be used to determine if there is any synergistic effect. Activation levels of the hormone receptors, EGFR and downstream components of EGFR signaling cascades can be analyzed.

While I have proposed a working model, there are other mechanisms that can be occurring either individually or simultaneously. GC may be interacting with and activating the hormone receptors leading to host signaling that facilitates GC transmigration. To examine this, activation levels of the hormone receptors can be examined in the absence and presence of GC. Receptor activation can be inhibited and the effect on GC transmigration determined.

The other possible mechanism involves the activation of host surface receptors, in addition to EGFR, by estrogen and progesterone. It has been shown that estrogen can cause activation of Erk 1/2 via EGFR activation [107] as well as Akt in an ER independent manner [199]. This shows that non hormone receptors can be activated by estrogen. Analysis of the activation levels of these surface receptors in the absence or

presence of hormones can be examined. Receptors with increased activity in the presence of hormones can undergo sequential inhibition of activity by either siRNA or inhibitory proteins and GC transmigration observed. Non-functional hormone homologs that can interact with, but not stimulate activation of, receptors can also be used and the effect on GC transmigration examined.

Appendices

A.1 Delta Opa

In addition to my two major research projects I also worked with the MS11 Δ opa strain that has been recently created in our lab by LeVan *et al* [200]. Opacity protein (Opa) is one of the surface molecules on *Neisseria gonorrhoeae* involved in pathogenesis and it mediates both adherence to and invasion into host cells [18]. MS11 strain expresses 11 distinct Opa proteins encoded by individual *opa* genes [201]. These proteins are capable of phase and antigenic variation at a high rate ($\sim 10^3$) [202]. These variations mean that a single GC colony can express multiple opa proteins and to varying levels. Under a dissecting microscope the presence of opa proteins causes an opaque, golden speckled appearance, but different Opas have differing degrees and shades of opacity. Due to the high rate of variation an accurate analysis of the role of Opa in GC pathogenesis may not truly have been obtained, since visually Opa lacking colonies may have low levels of Opa proteins present. As such our lab has constructed a MS11 strain that has all 11 genes either turned off or deleted, making a true Δ opa variant that cannot express Opa. Using this Δ opa MS11 strain I have analyzed three of the main stages of GC pathogenesis in the HEC-1-B cell line.

A.1.1 Lack of Opa affects GC invasion into both non-polarized and polarized epithelial cells

The role of Opa in GC adherence and invasion has been previously examined in non-polarized cells and it was determined that the presence of opa was associated with

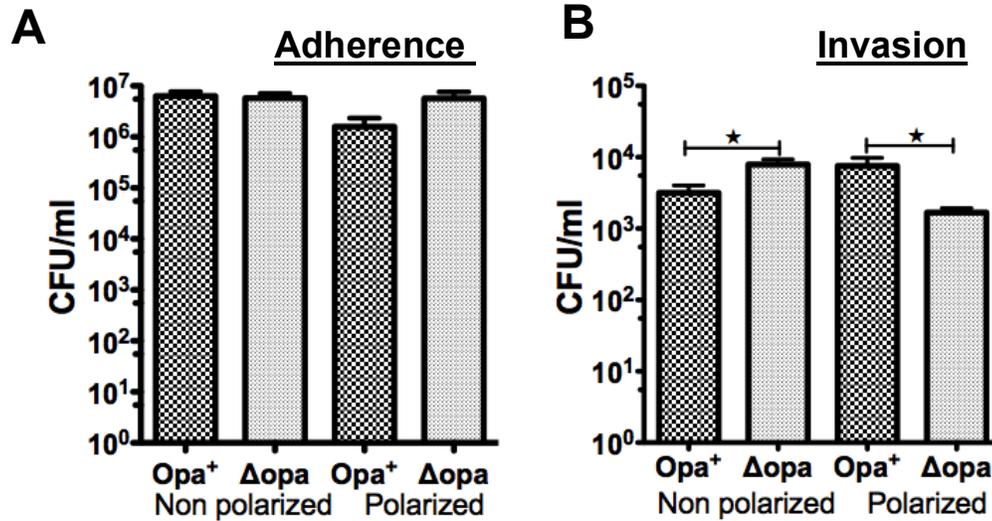


Figure. 24. Lack of opa affects GC invasion into cells. Non-polarized and polarized HEC-1-B cells were exposed to wild type or Δ opa GC for 3 h or 6 h. At 3 h the cells were washed, lysed and plated to enumerate associated GC (A). 6 h post inoculation cells were exposed for 2 h to gentamicin and then lysed to determine invaded bacteria (B). Shown are the average CFU (\pm S.D.) from three independent experiments. *, $p \leq 0.05$.

increased adherence and invasion [18, 203]. These results reinforced the clinical observations that the majority of GC recovered after infection express Opa. Even when volunteers were inoculated with phenotypically Opa⁻ GC mainly Opa⁺ GC were recovered [204, 205]. Using the Δ opa variant of MS11 there was no change in adherence to either non-polarized or polarized epithelial cells (Fig. 24A). However, there was an increase in invasion in non-polarized cells, while polarized cells showed a decrease in invasion (Fig. 24B).

A.1.2 GC transmigration was increased in the absence of Opa

Observing the later stage of pathogenesis, transmigration, there was an increase in transmigration across the polarized epithelial monolayer by GC lacking Opa (Fig 25). There were approximately 10 fold more Δ opa bacteria that transmigrated compared to their Opa⁺ counterparts. The majority of gonococcal research has been performed in non-polarized cells, and observations from these cells have been assumed to represent the infection process. As we perform experiments in polarized cells, our lab is observing that some phenomena assumed to be universal for GC pathogenesis are not observed when polarized cells are infected. In some cases the opposite effect is observed. The lack of Opa would reduce the strength of GC-GC interactions and might make it easier for individual GC to be endocytosed. The 6 h time point would not be enough to facilitate exocytosis, so the bacteria would be observed in increased numbers within the cell. The increased transmigration across polarized cells coupled with the observed increased invasion in non-polarized cells suggests a possible paracellular route for GC transcytosis. The reduced strength of the microcolony interactions would make it easier for gonococci

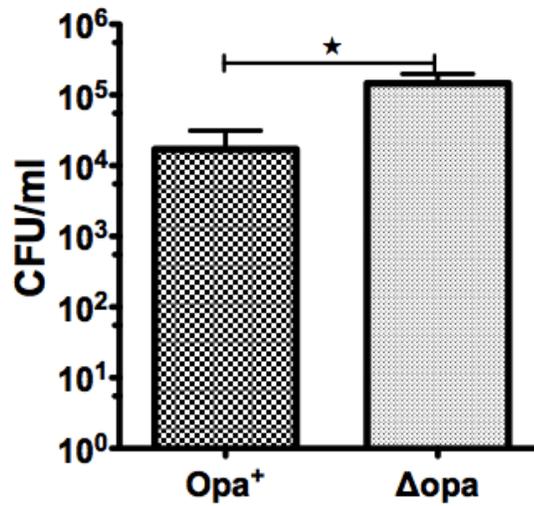


Figure. 25. Increased GC transmigration in the absence of Opa. Polarized HEC-1-B cells were exposed to O⁺ or Δopa GC for 6 h and the basal media collected and plated to determine transmigrated GC. Shown are the average CFU (±S.D.) from three independent experiments. *, p ≤ 0.05.

to pass between the weakened junctional complex. If the bacteria are crossing the monolayer via this means, then there are fewer bacteria available to invade the cells.

A.1.3 Predominantly Opa negative bacteria were recovered after transmigration

Finally, for invasion the majority of GC recovered were of the same Opa phenotype as the inoculum (Fig. 26). However for transmigration mainly phenotypic Opa⁻ GC were recovered from the basal compartment, regardless of whether Opa was present or absent in the inoculum (Fig. 26). EGTA was used as a control that destroyed the junctional complex so that some of the bacteria transmigrated via the paracellular route. EGTA treated cells were inoculated with Opa expressing GC and while most of the recovered GC lacked Opa, there was a significant amount of Opa expressing GC.

These results suggest that the lack of Opa may make GC more invasive and might play a role in complicated forms of the infection. HEC-1-B cells do not express CEACAM, a known Opa receptor, so this fact must be taken into account when analyzing these results.

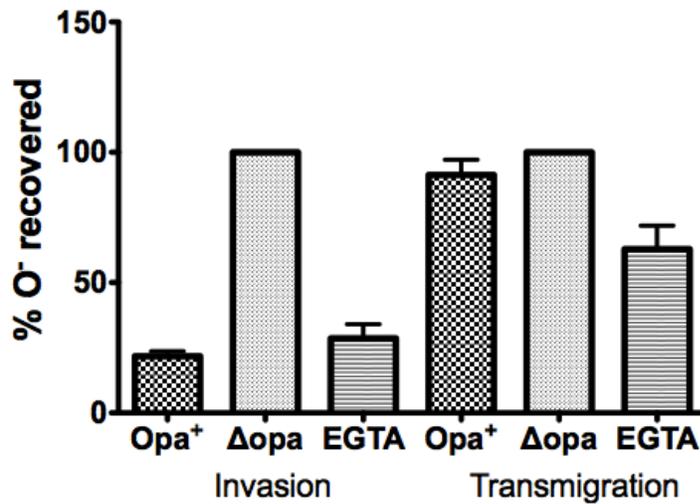


Figure. 26. Opa negative bacteria recovered after GC transmigration.

Polarized HEC-1-B cells were exposed to O⁺ (in the absence or presence of EGTA) or Δopa GC for 6 h and the phenotype of invaded and transmigrated GC visually determined using a dissecting microscope. The % of Opa lacking bacteria recovered from two independent experiments are reported.

A.2 Layman's Summary

Gonorrhea, caused by the bacteria *Neisseria gonorrhoeae*, is a common sexually transmitted infection (STI). A high percentage of women who are infected show no symptoms, thus leaving the infection untreated and increasing the likelihood of complications such as infertility. Recent observations of increased co-infection of gonorrhea with HIV make it a significant public health threat. In the present study, we show that live gonococci (GC) position themselves at the apical junction that keeps neighboring epithelial cells tightly linked. They cause junctional proteins (ZO-1, occludin and β -catenin) to disassociate from the apical junction, reducing the functional integrity of the junction. GC increase the activation of a common host surface receptor, epidermal growth factor receptor (EGFR), and the phosphorylation of β -catenin. If the kinase activity of EGFR is inhibited, not only does the disassociation of β -catenin from the junction reduce, but the number of bacteria that migrate across the protective epithelial layer also decreases. These results reveal for the first time that GC can weaken the protective epithelial layer via a host cell receptor, suggesting a new mechanism utilized by GC to cross the protective epithelial layer. The presence of the female sex hormones estrogen and progesterone, increase GC transmigration across the polarized monolayer. This increase does not appear to be mediated by 'classical' hormone receptor signaling, since inhibition of this pathway has no effect on the number of GC that transmigrate.

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