ABSTRACT

Title of Document: INTERACTIONS OF *NEISSERIA GONORRHOEAE* WITH EPITHELIAL CELLS

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Neisseria gonorrhoeae (GC) infects epithelial cells lining the female reproductive tract. The majority of GC infections in women are asymptomatic, which can lead to chronic inflammation and serious complications, including pelvic inflammatory disease, disseminated gonococcal infection, and infertility. GC is known to induce signaling in epithelial cells, including the activation of epidermal growth factor receptor (EGFR) and Ca^{2+} flux; however, the mechanism by which the signaling leads to GC infection is unknown. Using human cervical tissue explants and epithelial cell lines, my thesis research reveals that GC interactions with polarized epithelial cells induce EGFR-dependent Ca^{2+} flux, which activates and mobilizes the actin motor protein non-muscle myosin II (NMII). The Ca^{2+} flux and NMII activation are required for GC-induced disassembling of the apical junction and GC transmigration across epithelial cells, but not GC adherence and invasion. In addition, Opa proteins expressed on GC interfere with GC-induced NMII activation and apical junction

disruption, and alter GC interactions with the apical surface of the epithelium, consequently inhibiting GC transmigration. Thus, GC causes disruption of the epithelial barrier by inducing Ca^{2+}-dependent activation of NMII, and Opa phase variation modifies infection mechanisms by regulating perijunctional actomyosin remodeling. To understand how GC establishes infection in the female reproductive track, I examined GC infection in human cervical tissue explants as well as non-polarized and polarized epithelial cells in culture to mimic the non-polarized squamous ectocervical epithelial cells and the polarized columnar endocervical epithelial cells. My results show that GC interaction induces differential remodeling of the actin cytoskeleton in non-polarized and polarized epithelial cells of human ecto- and endo-cervical tissue explants and in culture. This differential actin remodeling is dependent on the activation and redistribution of NMII and leads to different changes in the morphology and functionality of epithelial cells. These results suggest that the polarity of epithelial cells at different anatomic locations of the female reproductive tract alters the mechanisms by which GC establishes the infection.

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By

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Dedication

I dedicate this dissertation to my parents, Yunn-Ming Wang and Hsin-Ti Lu, and my grandmother, Pei-Ying Lu-Hsu who always support me.

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List of Abbreviations

AJ adherens junction

ASM acidic sphegomyelinase

CEACAM carcinoembryonic antigen-related cell adhesion molecule

DAG diacylglycerol

DGI Disseminated gonococcal infection

EGFR epidermal growth factor receptor

ER endoplasmic reticulum

ERK extracellular signal related kinase

FBS fetal bovine serum

FRT female reproductive tract

GCK gonococcal media base

GC *Neisseria gonorrhoeae*

HEC-1-B human endometrial adenocarcinoma cell line

HSPG heparan sulfate proteoglycans

IgA immunoglobulin A

IP3 inositol triphosphate

JAM junctional adhesion molecule

LOS lipooligosaccharide

MAPK mitogen activated protein kinase

MEK1/2 MAPK kinase

MLC myosin light chain

MLCK myosin light chain kinase

MOI multiplicity of infection

NMII non-muscle myosin II

Opa opacity-associated proteins

PC-PLC phosphatidylcholine-specific phospholipase C

PI3K phosphoinositide 3-kinase

PID pelvic inflammatory disease

Pil pili

PIP2 phosphoinositol 4,5 bisphosphate

PIP3 phosphoinositol 3,4,5 bisphosphate

PKC Protein Kinase C

PLCγ phospholipase-gamma

pMLC phosphorylated myosin light chain

ROCK Rho-associated kinase

RTK receptor tyrosine kinase

SH2 Src homology 2

T84 human colorectal adenocarcinoma cell line

TEER transepithelial resistance

TJ tight junction

ZO zonula occludens

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Chapter 1: Introduction

1.1 Neisseria gonorrhoeae infections

One of the oldest human diseases recorded is gonorrhea, a sexually transmitted infection that affects approximately 80 million individuals worldwide. While most infections occur in developing nations, there are over 300,000 cases reported annually in the United States, making gonorrhea the second most commonly reported infectious disease in the U.S. [1]. Demographically, the groups most affected by this sexually transmitted infection are teenagers and young adults. Initially, the infection was believed to predominately affect males; however, recent statistics have shown that the infection rate in females is higher [2].

The causative agent of gonorrhea is the human obligate pathogen Neisseria gonorrhoeae (GC), which is a gram negative, diplococcal bacterium. GC primarily infects the urogenital epithelia, and thus the initial sites of infection are the urethra and uterine cervix in males and females respectively. In males the disease is usually symptomatic, which presents as urethral discharge or abnormal exudates [3]. These symptoms enable early diagnosis and treatment. The patient can be treated with antibiotics from the cephalosporin family such as ceftriaxone or cefixime [4]. In contrast, female infection generally shows no visible manifestation of the disease. [3]. This prohibits early diagnosis and treatment consequently causing extended colonization and chronic infection, which may lead to pelvic inflammatory disease

(PID) or even disseminated gonococcal infection (DGI). Consequences of PID include scarring of the reproductive organs, which may result in chronic pelvic pain, ectopic pregnancy and/or infertility. The significance of gonorrhea is further highlighted by the findings that gonococcal infection increases the risk of HIV infection and co-infections of other sexually transmitted pathogens [1].

GC has gradually developed resistance to each of commonly-used antibiotics. In the past years, the development of fluoroquinolone resistance has made cephalosporins the only available antibiotics for gonorrhea treatment [5]. Recently, declining susceptibility to cefixime, a specific type of cephalosporin, resulted in a change in treatment from single antibiotic to dual therapy, using ceftriaxone plus either azithromycin or doxycycline [6]. The emerging threat of cephalosporin resistance, in combination with subclinical gonorrhea in women, highlights the need for further research on gonorrhea.

1.2 Neisseria gonorrhoeae and virulence factors

To establish infection, GC interacts with the epithelial cells of the genital tract via gonococcal surface molecules. The main surface components of GC are pili, porin, opacity-associated (Opa) proteins, and lipooligosaccharide (LOS). The type IV pili complex, consisting of the major protein Pil E and minor protein Pil C, is responsible for initiating interaction with the host cell. [7, 8]. Opa proteins, which exist in eleven variants that are encoded by eleven different genes, are responsible for intimate interactions of GC with host cells. Porin, the most abundant surface protein on the GC

surface, forms anion selective channels and is required for GC survival. Moreover, gonococcal porin can be transferred from the bacteria to human epithelial cells, which has been shown to induce transient calcium influx [9]. Finally, the surface oligosaccharide LOS can be sialylated in the host, which protects the bacterium from recognition by the host immune factors, including bactericidal antibodies and complement, allowing GC to survive within the host [10].

The pathogenic members of the Neisseriaceae family undergo both phase and antigenic variation, which enables them to evade the host defense mechanisms and propagate within the host. [11]. For example, the expression of eleven Opa proteins can be switched on or off randomly (phase on and off) thereby changing GC-GC and GC-host cell interactions [12]. The signal peptide DNA of each opa gene contains a repetitive pentamer sequence (5"-CTCTT-3"). Depending on the number of pentamer sequence repeats, the translational reading frame of an opa gene may be shifted, turning each opa gene translation on or off [13]. It has been postulated that the phase variation of these surface molecules [14-16] renders GC the capability of infecting various anatomic locations of epithelium and generating different pathological conditions of the infection.

1.3 Neisseria gonorrhoeae interaction with human epithelium

In multicellular organisms, epithelia provide an interface that separates the individual from the environment. Epithelia serve as the first line of defense against various

stimuli such as mechanical damages, chemical stimulants, toxins, and microbial pathogens such as GC.

Based on the current literature, GC establish infection in the epithelium by three interrelated processes: 1) initial attachment and close association between the surfaces of the bacteria and the host epithelium, 2) invasion of the bacteria into the host epithelium and survival of the bacteria within the cell, and 3) dissemination into deeper tissues [17].

The initial contact of the bacteria with host epithelial cells normally occurs via type IV pili. Pili retraction brings the bacteria close to the host cell surface, allowing anchorage and the formation of microcolonies at sites of attachment [18]. Opa proteins and LOS at the bacterial surface then become involved in intimate interactions with host cell receptors. The interaction of GC with the host cell surface induces elongation of the host microvilli that subsequently surround the bacteria. The Opa proteins have been reported to interact with different host cell receptors including Heparan Sulfate Proteoglycan receptors and CD66 family receptors [19-23]. The type of Opa expressed determines the targeted host receptor(s) subsequent signaling triggered, and types of host cell responses [17, 18, 24, 25]. The phase variable surface carbohydrates of LOS is also capable of binding to host asialoglycoprotein receptors and assists with gonococcal uptake [26]. Our lab has previously shown that LOS containing lacto-*N*-neotetrose structures is required for gonococcal invasion with Opa expression turned off [27].

Multiple interactions between the bacteria and host cell trigger various signaling and cellular apparatus in host epithelial cells, from actin rearrangement to engulfment of the bacteria into vacuoles. Previous studies suggest that GC are able to survive within host cells after entry. Porins are capable of making pores in the host cell membrane, which facilitates GC survival within epithelial cells by potentially altering host signaling [28]. IgA protease secreted by GC has been implied to enable GC to escape from vacuoles [29], while other studies suggest GC remain within a vacuole once internalized by epithelial cells. While other studies suggest GC remain within a vacuole once internalized in the cell. Regardless of whether GC remain within the vacuole or escape to the cytoplasm, they appear to be capable of replication [30].

In summary, gonococcal adherence and invasion are two critical processes for establishing infection. However, adherence and invasion may not be sufficient for GC to survive and establish chronic infection due to the fact that the female genital tract sheds epithelial cells during the menstrual cycle. Transmigration, which allows gonococcal penetration into deeper tissue - is probably required for GC to persist and cause disseminated disease.

GC transmigration across epithelial cells lining the surface of the female genital tract was first suggested by *Ward et al*. Using human fallopian tube organ-culture as a GC infection system, GC were found in both the epithelial layer and subepithelial connective tissue [31]. In order to reach subepithelial tissues without causing

symptoms, GC need to cross the epithelium first. There are two possible ways for GC to transmigrate across epithelium: intracellular or paracellular pathways. The intracellular pathway involves the transcytosis of apically invaded GC through the cytoplasm of epithelial cells and the escape of transcytosed bacteria from the basal surface near subepithelial connective tissue. The paracellular pathway involves GC passing through the space between epithelial cells to reach subepithelial tissues.

Several studies utilizing cell lines have confirmed Ward's discovery of transmigration. However, most previous studies only considered the intracellular transmigration pathway, based on the ability of GC to undergo receptor-mediated invasion [32]. *Spence et al*. suggested that the lutropin receptor expressed on polarized HEC-1-B cells facilitated gonococcal transmigration in a contact-induced manner [33]. The lutropin receptor may provide a link between PID and DGI with the beginning of menses, as this is the period when the expression of lutropin receptors peak in the fallopian tubes. This study implied that the adhesin protein L12 expressed by an F62 GC strain enhanced transmigration across the polarized layer via binding to lutropin receptors [33]. Previous studies also showed that different gonococcal strains expressing various surface molecules induce transmigration with different kinetics and in various time frames [34, 35]. These data showed that GC has developed diverse mechanisms to invade and survive within hosts, by manipulating existing host cellular apparatus [36]. The adherence and invasion mechanisms utilized by GC have been extensively studied; however, the mechanisms by which GC transmigrate across epithelial cells have not been elucidated. Moreover, the GC-epithelium interaction

studies conducted thus far haven't represented the GC infection *in vivo* due to the lack of a model for epithelium in the female reproductive tract.

1.4 Epithelium and apical junctions in the female reproductive tract

The epithelium lining on the FRT has a crucial role in reproduction and immune defense. There are two types of epithelia in the FRT - multiple layers of squamous epithelium and a single layer of columnar epithelium. The two types of epithelia consist of non-polarized and polarized epithelial cells, respectively [37]. The lower female genital tract including the vagina and ectocervix consists of non-polarized squamous epithelial cells, and the region from the endocervix to the fallopian tubes contains polarized columnar epithelial cells [38-40]. The two different epithelia are morphologically and functionally different based on their polarity.

In order to build epithelial polarity, the space between adjacent epithelial cells needs to be sealed. The adhesion molecules on neighboring epithelial cells interact with one another, which form regulated junctional complexes in the lumen side of the paracellular space, the apical junction, thus separating the environment apart from the host [41].

The apical junction, which seals the space between adjacent epithelial cells, consists of two major types of cell-cell junctions that have a role in sealing the space between adjacent epithelial cells are tight junctions (TJ) and adherens junctions (AJ). The apical junction divides the plasma membrane of epithelial cells into an apical side -

free of attaching to other cells but facing the environment, and a basal side - which is anchored to the extracellular matrix and other cells in the subepithelial tissues. Because these two sides are functionally and structurally different, the epithelium becomes polarized. The main components of the apical junction can be divided into three main categories. First, transmembrane/integral proteins that are involved in direct homo- or hetero-transinteraction with each other between adjacent cells, thus serving as the gate of paracellular space. Second, the peripheral associated scaffolding/adaptor proteins that are involved in organizing the proteins at the junction and linking these proteins to the actin cytoskeleton. Finally, the signaling adaptor proteins that link signal transduction to the assembly and disassembly of the junctional complex [42, 43].

The tight junction contains the transmembrane proteins occludin, claudin and junction adhesion molecule (JAM). The adherens junction that is located on the basolateral side of the tight junction contains a transmembrane protein, E- cadherin. The TJ and AJ complexes are linked to the actin cytoskeleton by adaptor proteins Zonula Occluden (ZO) and α/β-catenin, respectively. These links are tightly regulated by various cellular signaling proteins [44, 45].

In the female reproductive tract, non-polarized stratified squamous epithelial cells cover the vagina and ectocervix epithelium [46]. The squamous epithelial cells form multilayers with AJs gradually appear, starting from the third to forth layer of squamous epithelial cells from the luminal surface (Fig. 1). The top few layers of epithelial cells of the ectocervix are cornified, filled with glycogen and do not show

any specialized cell junctions. Instead, they form loose contact with adjacent cells by a short protrusion. This differs from AJs between neighboring cells, which arise in the suprabasal layers of the ectocervix. The epithelial cells at the bottom layer express higher levels of the junction proteins claudin, JAM, and ZO-1 than those in the top layer, while the proliferation level of epithelial cells at the top layer decreases compared to that of the bottom layers. However, different from the apical junctional location of ZO-1 in polarized epithelial cells, ZO-1 distributes as puncta along cell-cell contacts as well as in the cytoplasm of layered squamous epithelial cells [47] (Fig. 2). The penetration of fluorescently labelled IgG through epithelial cells to the suprabasal layer indicates that vaginal and ectocervical epithelia are permeable [47].

The surface of the endocervical canal is covered by a single layer of columnar epithelial cells, which invaginate to form a complex system of clefts (Fig. 1). Endocervical epithelial cells synthesize mucus and other substances and package them into secretory vesicles for secretion [46]. Endocervical epithelial cells are organized as a monolayer via the classical apical junctional complexes, including claudin, JAM and ZO-1, similar to other mucosal surface. In the apical region, neighboring endocervical cells are joined by tight junctional protein complexes, which effectively seal the apex of each cell to prevent intercellular passage of luminal contents. Below the tight junctions, adherens junctions, consisting of E-cadherin, form multiple adhesive bands between adjacent cells, helping to maintain the stability of the apical junction (Fig. 2). Function studies showed that the endocervical polarized epithelial cell layer is able to exclude molecules in the lumen based on the

prevention of the fluorescently labelled IgG passage [47]. Because the epithelium of the lower female genital tract is the primary site of GC, an in-depth understanding of gonococcal interactions with epithelial cells and the impact of the interactions on the structure and function of epithelial cells is crucial for delineating the mechanism by which GC cause gonorrhea and complications in the female reproductive tract. So far, there are only limited studies that examine the relationship between the epithelial cells in the female reproductive tract and GC infection, focusing on characterizing the cellular response in human cervicovaginal tissue or derived cell lines to GC infection [48-52]. Additional studies are required to shed light on the cellular and molecular mechanisms by which GC infect the two different types of cervical epithelial cells.

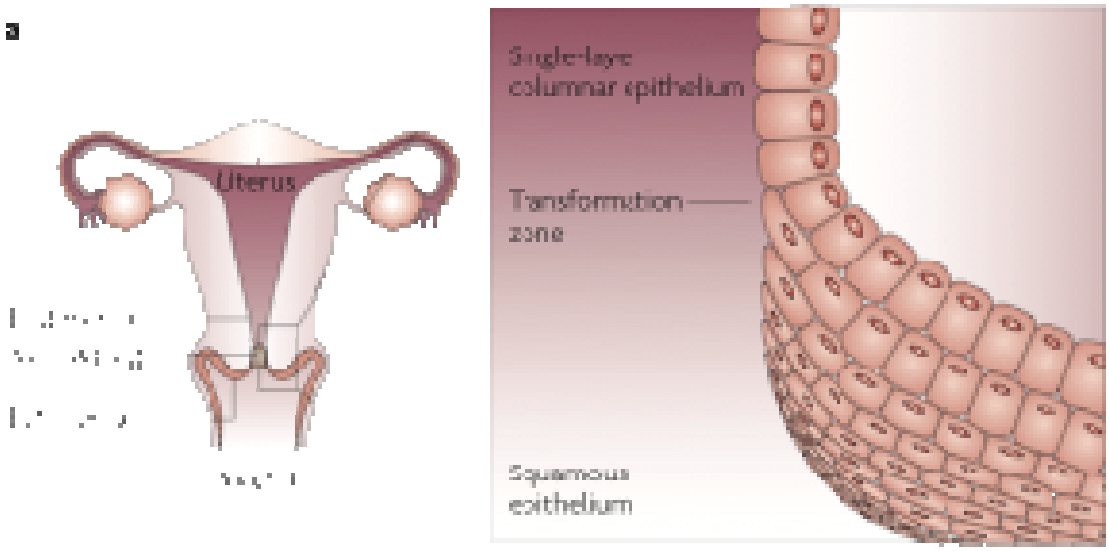


Figure 1. Cervical epithelium [53]

In the female reproductive tract, the vagina and ectocervix consist of the non-keratinized squamous epithelium.The endocervix and above consist of the single-layer columnar epithelium. The endocervical canal is filled with mucus, providing a barrier against the ascent of pathogens.

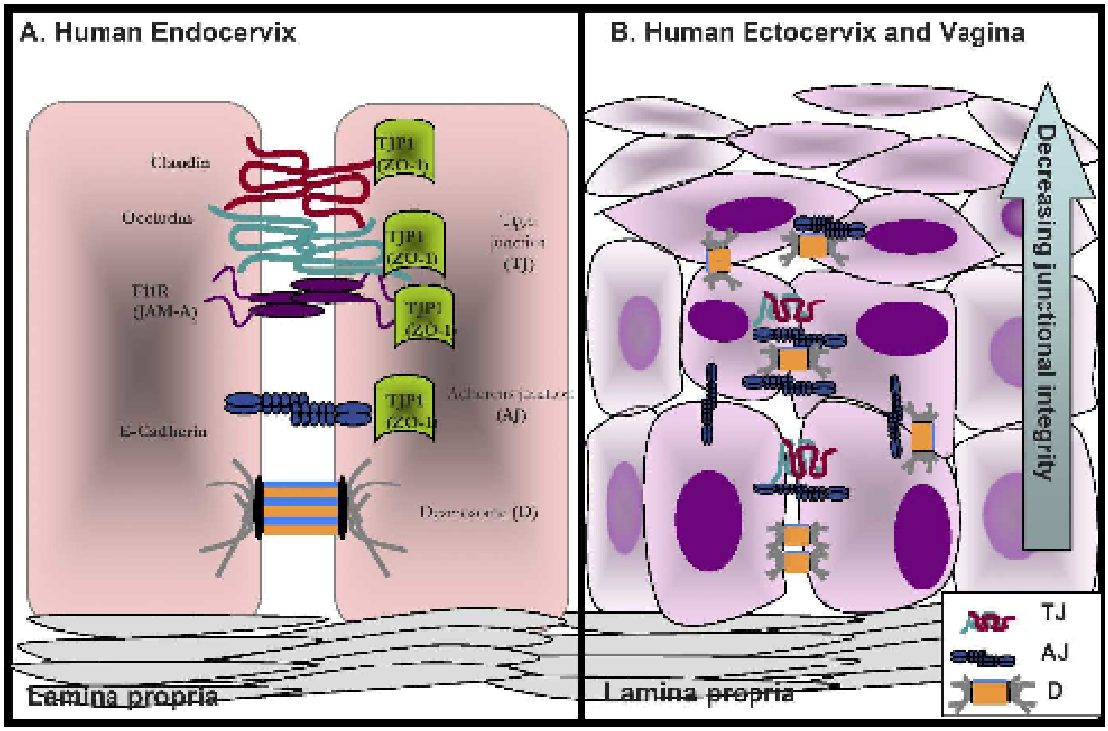


Figure 2. Localization of junction molecules in the female lower reproductive tract [47]

A) The endocervical epithelium contains classic junctions. The tight junctions are located near the apical surface; they seal the epithelium and maintain cellular polarity. Adherens junctions are located directly below the tight junctions and are primarily responsible for cell-cell adhesion. E-cadherin is the common transcellular component of all epithelial adherens junctions and is anchored to the actin cytoskeleton by catenins. B) The most robust junctions in the stratified squamous epithelium of the ectocervix and vagina lie in the parabasal epithelium, just above the basal layer in contact with the basement membrane. Adherens junctions are relatively abundant. The integrity of the junctions progressively lessens as epithelial cells are pushed toward the apical surface, where they become cornified, lose all cellular contacts, and are sloughed into the lumen

1.5 Apical junction regulation

The apical junction plays two significant roles in generating and maintaining the polarity of epithelial cells. The 'fence' function of the apical junction prevents proteins and lipids in the apical and basolateral membranes from moving laterally, thereby maintaining the functional polarity of the two surfaces. The 'gate' function regulates the paracellular permeability of epithelial cells, preventing small molecules, irons, and organisms such as mucosal pathogens, from crossing the polarized 1 complexes regulates the fence and gate function of the apical junction [54].

Previous studies have suggested several cellular mechanisms to regulate the function of the apical junction. First, the transmembrane junction proteins can undergo rapid endocytosis and recycling. The endocytosis of junctional proteins is mediated by either clathrin- or caveolin-mediated endocytosis. A TNF-related cytokine - LIGHT, has been shown to induce occludin redistribution from tight junction to caveolin-1-containing vesicles [55]. Second, the phosphorylation of junctional proteins can attenuate interactions between the scaffolding proteins and transmembrane proteins in the junctional complex. For example, tyrosine phosphorylation of the C-terminal domain of occludin weakens its interaction with ZO-1 [56]. Third, the apical junction can be regulated by the dynamics of perijunctional actin rings. Myosin-mediated actin contraction can transiently increase the permeability of the apical junction or induce the disassembly of the junctional complex [45, 57, 58]. It has been shown that GC are able to disrupt tight and adherens junctions [50], however the molecular mechanism is still unclear.

1.6 The actin cytoskeleton

The actin cytoskeleton is an apical junction regulator and also required for GC infection. Actin in polarized epithelial cells concentrates at the apical surface, forming a perijunctional actin ring and microvilli protruding into the lumen [45]. The perijunctional actin ring is linked to the apical junction by ZO-1 to the TJ and α/β-catenin to the AJ. Both TJ and AJ are regulated in part by the perijunctional actin ring. On the basolateral side of polarized epithelial cells, there are stress fibers and focal contacts with the extracellular matrix. The polarity of the actin cytoskeleton was shown to be important for the polarity of epithelial cells [45, 59]. Disruption of the actin cytoskeleton by cytochalasin D increases the permeability of cultured endothelial cells, whereas treatment with phallacidin, an actin stabilizer, prevents agonist-mediated barrier dysfunction [60]. In contrast, in non-polarized epithelial cells, actin concentrates at the cell edge to form cortical actin networks and support lamellipodia and filopodia. Actin remodels in response to environmental stimuli such as mechanical and chemical signals from pathogens [61].

The actin cytoskeleton is one of the host factors involved in GC infection. Inhibition of actin rearrangement by cytochalasin D reduces GC invasion into host cells to nearly zero [62, 63]. Studies accumulated over the past 20 years demonstrate that actin rearrangement occurs, and that this rearrangement is required for GC infection. However, the nature and magnitude of actin rearrangement varies based on the GC strain and the type of epithelial cell [64]. How GC signal to the actin cytoskeleton in host cells is unknown. Based on what we know about the regulation of actin

dynamics, it is likely that GC activate signal via host receptors either directly or indirectly [65].

The major surface molecules Opa, Pili, and LOS play roles in inducing actin rearrangement by binding to host receptors [11]. In primary human urethral epithelial cells, Opa-dependent GC invasion is accompanied by rearrangement of the actin cytoskeleton that drives microvilli extension to surround adhered GC [63, 66]. In human epithelial cells A431, GC induce cortical plaques and extension of microvilli at the adherent site in a pili-dependent manner [67]. LOS-dependent induction of actin mobilization was also observed in infected ME180 cervical epithelial cells [27]. Using primary cervical cells, *Edwards et al*. showed that gonococcal adherence induced actin-dependent morphological changes such as the formation of filopodia and lamellipodia, as well as membrane ruffling. These results are consistent with the observation that actin-associated proteins, such as vinculin and ezrin, are accumulated at cell membrane protrusions in contact with GC [62].

In addition to GC, other microbial pathogens, e.g., *Listeria, Salmonella, Shigella, Yersinia,* and *Bartonella* can manipulate the host actin cytoskeleton to facilitate invasion and intracellular survival. In particular, *Listeria, Shigella* and *Rickettsia* can harness the actin cytoskeleton for their movement in the cytoplasm of host cells [65]. Most of the bacteria secrete effectors that regulate the host actin cytoskeleton [68]. However, no GC secreted protein that regulates actin has been identified. One possible mechanism for GC to modulate the actin cytoskeleton is by triggering host

signaling. However, host signal pathways and actin regulators induced by GC that are responsible for GC-induced actin rearrangement remain undefined.

1.7 Non-muscle myosin II

One critical component of the perijunctional actin structure as an actin regulator in polarized epithelial cells is non-muscle myosin II (NMII). Activated NMII regulates the structural and functional integraty of the apical junction by contracting the perijunctional actin cytoskeleton. Actomyosin contraction is also one of the mechanisms by which cells and pathogens to regulate paracellular permeability. In addition, NMII plays essential roles in actin-mediated cell migration, cell adhesion, and tissue architecture. For example, in migrating cells, actin rapidly polymerizes in the front forming protrusions, while NMII contracts in the opposite side of the cell moving front, which induces actin depolymerization [69] and retract cell membrane. The synergy of the actin polymerization and NMII-mediated actin contraction and depolymerization drives cell migration [70]. Another example is phagocytosis/endocytosis where NMII facilitates the formation of endocytic vesicles by strengthening the actin cytoskeleton surrounding the vesicle [71].

NMII is one of the actin motors and belongs to myosin super family. NMII consists of two heavy chains of 200 kDa and four light chains of 20 kDa, the latter of which are called essential (MLC1) and regulatory light chains (MLC2/RLC), respectively. The heavy chains dimerize via the coiled-coil region, termed rod or tail. The ATPase is located in the N-terminal forming a large globular head projecting from the rod

domain. The MLC1 and MLC2 are structurally related to calmodulin, and bind to the heavy chain at the distal region of the head domain [44] (Fig. 3). NMII has three functional subdomains: (1) the motor domain formed by the head domain of the heavy chain that interacts with F-actin and binds and hydrolyzes ATP; (2) the neck domain to that light chains or calmodulin binds; and (3) the tail domain which serves to anchor and position the motor domain enabling it to interact with F-actin [72] (Fig. 3).

Actomyosin contraction is induced by phosphorylation of MLC2/RLC. The phosphorylation of MLC2/RLC has long been implicated in TJ regulation [73, 74], and has recently been shown to be sufficient for triggering TJ and AJ disassembly [70, 75, 76]. Phosphorylation on Ser19 residue of the MLC2/RLC is responsible for the regulation of NMII activity [77, 78]. The phosphorylation activates the ATPase and contractile activity of NMII [79]. This phosphorylation event has been attributed to several different kinases, suggesting that NMII may be subject to regulation by multiple signal transduction pathways. At present, two kinases are known to be responsible for the phosphorylation of the MLC2/RLC: Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK) and Rho-associated kinase (ROCK) [73, 78] (Fig4).

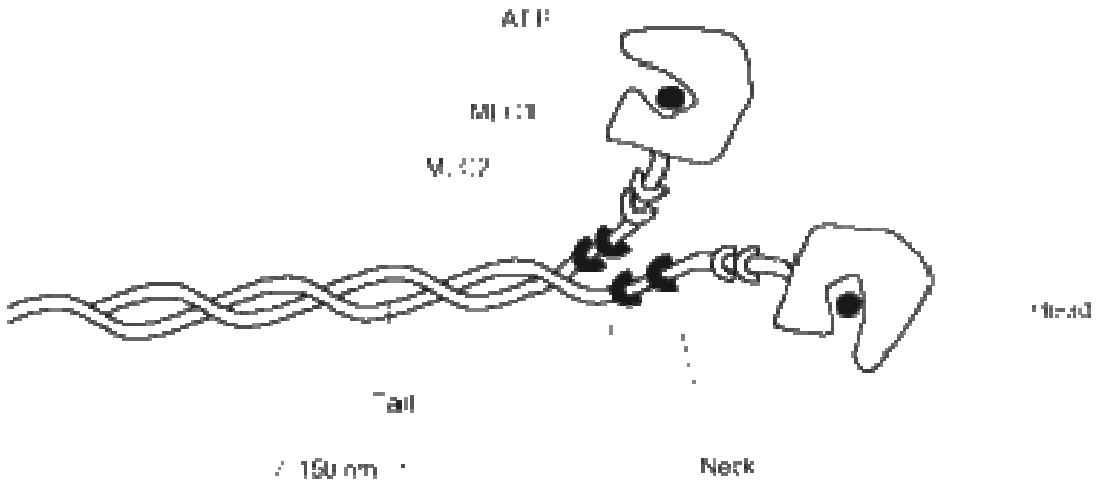


Figure 3. Model of myosin II [44]

Myosin II is composed of two heavy chains that form a parallel structure organized as a coiled-coil rod from which large NH2 terminal globular heads project. Two light chains are found around each neck: an essential light chain (MLC1) and a regulatory light chain (MLC2).

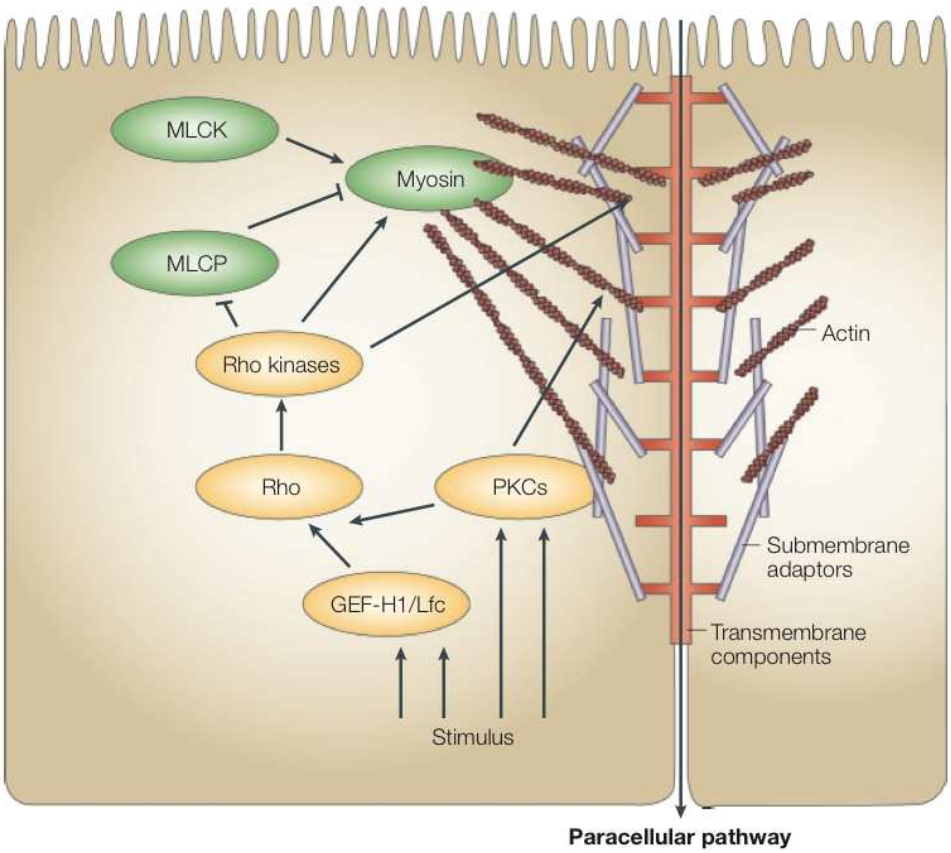


Figure 4. Regulation of junction by Rho kinase and Myosin light chain kinase [80]

A schematic representation of the signaling proteins in the regulation of apical junctions (TJs and AJs), and how they might regulate paracellular permeability. Transmembrane components of apical junctions are shown in red. The submembrane adaptors of the junctional plaque are indicated in purple. Several adaptors function as linkers to the actin-based cytoskeleton. Regulation of the actin cytoskeleton by changing the activities of myosin light-chain phosphatase (MLCP) and myosin light-chain kinase (MLCK) is a mechanism of regulation of paracellular permeability. The other pathway of junction regulation is signaling by the small GTPase Rho. This Rho-dependent pathway involves Rho kinases as well as Rho-specific guanine nucleotide exchange factors (GEFs).

The involvement of NMII in microbial pathogenesis has been indicated by the finding of that inhibition of MLCK prevents or reverses the loss of TJ barrier function induced by bacterial and parasitic infection [81-83]. MLCK-driven MLC phosphorylation is a regulating target of various signal transduction pathways that lead to reductions in the barrier function of the apical junction in response to various stimuli, such as growth factors and mechanical stress [84]. The main activator of MLCK is Ca^{2+}-calmodulin complex, which wraps around the calmodulin-binding region of MLCK and causes the conformational change and activation of MLCK. The activated MLCK can then phosphorylate MLC2/RLC for NMII activation [85]. Rho family GTPases act as molecular switches, binding to and signaling through effector proteins when in the GTP-bound form. ROCK, one of the effector proteins of Rho-GTPase, activates NMII via two mechanisms. First, it phosphorylates MLC2/RLC, which leads to actomyosin contraction [78]. Second, it inhibits myosin light-chain phosphatase (MLCP) which dephosphorylates MLC2/RLC, consequently deactivating NMII [86]. Together, these mechanisms regulate the level and location of NMII activation (Fig. 4).

In summary, NMII plays an important role in regulating apical junctions and the actin cytoskeleton, and NMII is regulated via a calmodulin-dependent and the Rho-GTPase-dependent signaling pathways. However, we do not yet know whether and how GC utilizes NMII for disrupting the apical junction of epithelial cells in the female reproductive tract.

Previous studies have shown that microbial pathogens target NMII to regulate epithelial permeability [84, 85]. However, there is limited research on the regulation of the apical junction of epithelial cells in the female reproductive tract. An early report suggests a role for NMII in regulating paracellular permeability of cervical epithelium [87]. However, we do not know if GC can induce apical junction regulation through NMII and how GC-epithelial cells interaction initiate NMII activation.

1.8 Gonococci-induced host cell signaling

In order to regulate host cell machinery for successful infection, GC needs to initiate signaling within host cells. GC has been shown to induce various signaling pathways in host cells, including MAPK/ERK, phosphatidylcholine-specific phopholipase C (PC-PLC) and PI3K pathways. The downstream components of these signaling pathways, including Ca^{2+} flux, Akt kinase, and acid sphingomyelinase (ASM), as well as the Rho family GTPases, have also been reported to be activated in response to GC infection.

MAP/ERK activation has been reported in response to GC-epithelial cell interaction, and has been shown to be dependent on gonococcal pili. This pathway has been suggested to provide gonococcal infected host cells with cytoprotection by down regulating the pro-apoptotic proteins Bad and Bim [88] although another report

showed that GC-induced ERK activation is Opa and pili independent and may have a role in disrupting the junctions of epithelial cells [89].

PC-PLC has also been reported to be activated in cervical or urinary epithelial cell lines and primary fibroblasts upon gonococcal infection. It was suggested that PC-PLC contributes to gonococcal invasion by activating acid sphingomyelinase (ASM) [90].

It has shown that GC can induce PI3K by Opa-mediated interaction with carcinoembryonic antigen-related cell adhesion molecule (CEACAM). This signaling pathway contributes to GC internalization in epithelial cells, probably via activating Akt [91]. Akt has been correlated with PI3K dependent microcolony formation of GC on the epithelial surface, although Akt in GC-infected epithelial cells have been shown to be dependent on gonococcal phospholipase D (NgPLD) homolog and independent of PI3K [48, 92]. Both activation mechanisms may contribute to GC infection.

Although there are multiple signals downstream from major signaling pathways, the two that can mediate/regulate actin as well as junctions are Rho- family GTPase and Ca^{2+}.

Rho-family GTPase Rac1 and Cdc42 have been reported to contribute to GC-induced actin rearrangements. GC activates Rho-family GTPases probably through Src kinases or ASM [90, 93].

Ca^{2+} flux is an important and universal second messenger in eukaryotic cells [94-97]. It is well known that GC can induce both intracellular and extracellular Ca^{2+} influx. The porin (PorB) and PilT-mediated pilus retraction have been suggested to induce Ca^{2+} flux. PorB can form pores in the membrane of host epithelial cells, enabling rapid Ca^{2+} influx (~2 min) [94, 97], while pili retraction induces the release of Ca^{2+} from intracellular stores within ~10 min [95, 96]. It also has been shown that Ca^{2+} flux induced by pili retraction is dependent upon PorB-induced Ca^{2+} influx [9]. However, other signaling pathways, such as PLCγ, can also induce Ca^{2+} flux from the intracellular store in a later time point [98]. Ca^{2+} signaling may play an important role in the infection process by regulating signaling pathways.

In summary, various host signaling pathways are induced by GC-host cell interaction. However, there wasn't a defined signaling map shown to link GC-epithelium interaction to GC infection. A more complete signaling map will be needed from the initial signaling to the outcome in order to understand GC infection.

1.9 Gonococci and Epidermal growth factor receptors

The interaction of GC with host cells has been shown to mediate by GC-host receptor binding, which initiates host signaling pathways. It has been shown that GC-epithelial cell interaction induces the activation of epidermal growth factor receptor (EGFR), a receptor belonging to the ErbB family receptors that contain tyrosine kinases (RTKs)

in their cytoplasmic tails [99]. Recent studies from our lab showed that GC could induce tyrosine phosphorylation of EGFR in both polarized and non-polarized epithelial cells [51]. EGFR activation is not triggered by direct interaction of GC with EGFR; instead GC increases the production and surface cleavage of EGFR ligand. Studies from our lab have shown that GC induces recruitment of EGFR and ErbB2 to bacterial adherent sites in both non-polarized and polarized epithelial cells. Even though EGFR is preferentially localized at the basolateral surface of the polarized epithelial cells, apical inoculation of GC leads to the recruitment of EGFR to gonococcal microcolonies at the apical surface [51]. Inhibiting EGFR kinase using an inhibitor reduces gonococcal invasion into non-polarized endometrial epithelial cells HEC-1-B and cervical epithelial cells ME180 and GC transmigration across polarized HEC-1-B. These results indicate EGFR activation is required for gonococcal invasion in non-polarized epithelial cells [51] and transmigration across polarized epithelial cells [50].

There are three main signaling pathways downstream of EGFR: MAPK/ERK, PLCγ, and PI3K pathways (Fig. 5). Activation of the MAPK/ERK pathway is initiated by the docking of the adaptor protein Grb2 and/or Grb2/Shc to phosphotyrosines in the cytoplasmic tail of EGFR. Grb2 activates SOS, the guanine exchange factor of the small G-protein Ras. Ras in turn activates MAP kinase pathway, from Raf (MAPK kinase kinase) to MEK1/2 (MAPK kinase and ERK1/2 (p44/p42 MAPK). ERK1/2 can activate multiple transcription factors [100], leading to expression of genes that

are required for cell proliferation [45]. Phopholipase C - γ (PLCγ), a signaling molecule downstream of EGFR, cleaves phosphoinositol-4,5-bisphosphate [PI(4,5)P_2] to diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 activates IP_3-receptor-dependent Ca^{2+} release from the endoplasmic reticulum (ER), which in turn activates the ER Ca^{2+} sensor STIM. STIM induces Ca^{2+} influx via the Ca^{2+} channel ORAI1 in the plasma membrane [101], and Ca^{2+} signals for actin and actomyosin remodeling. For example, MLCK is activated by Ca^{2+}-bound calmodulin [98], and activation of gelsolin, an F-actin severing protein, requires the binding of Ca^{2+}. EGFR activates PI3K through the adaptor protein Gab1. PI3K in turn activates AKT/PKB (protein kinase B) by generating PI(3,4,5)P_3. The AKT/PKB pathway is one of the major signaling pathways promoting cell growth [102] (Fig. 5).

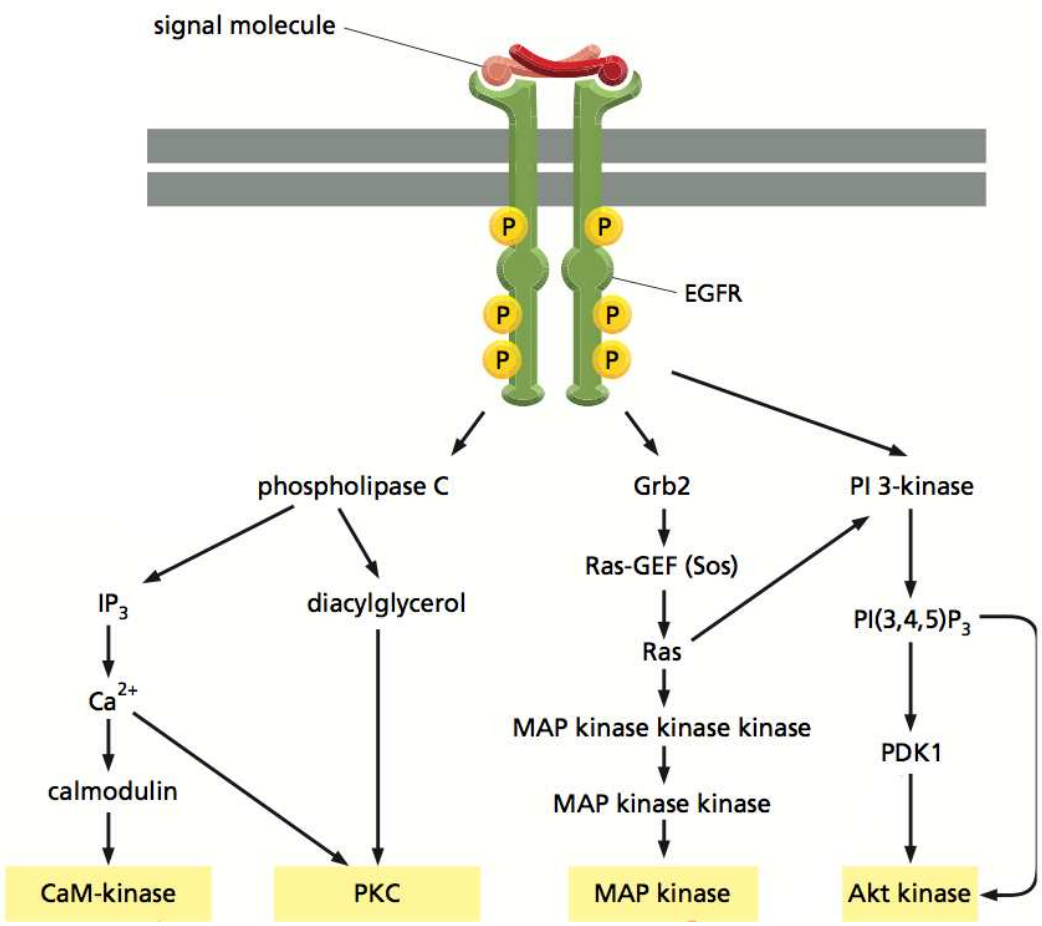


Figure 5. Three parallel intracellular signaling pathway activated by EGFR [Modified from *Molecular Biology of The Cell fifth edition*]

In this signaling map, the four kinases (shaded yellow) at the end of each signaling pathway phosphorylate target proteins.

Our preliminary studies found that GC-induced EGFR transactivation leads to the activation of the three downstream signaling pathways of EGFR. Among these three signaling pathways, PLCγ, but not ERK1/2 and PI3K, is likely involved in gonococcal invasion, as only PLCγ siRNA but not ERK and PI3K inhibitors inhibits gonococcal invasion. However, we do not know which of the EGFR downstream pathways is required for gonococcal infection in polarized epithelial cells and whether different signaling pathways are involved in different mechanisms for GC to establish infection and different complications.

EGFR is critical for regulating cell-cell junctions formed between neighboring epithelial cells. In polarized epithelial cells, it has been found EGFR activation can induce the disassembly of cell-cell junctions by inducing signaling towards junction protein disassociation and endocytosis. Previous studies have shown that EGF stimulation increases the cytoplasmic pool of tyrosine-phosphorylated β-catenin. The phosphorylation causes the disassociation of β-catenin from E-cadherin and perijunctional actin structures, consequently detaching the perijunctional actin support from E-cadherin [103, 104]. EGFR activation leads to caveolin-mediated endocytosis of E-cadherin as well as Snail-mediated transcriptional repression of E-cadherin [105]. In addition, EGF stimulation has been shown to induce cellular redistribution of the TJ proteins claudin and ZO-1 and a reduction in the TJ transepithelial resistance in polarized Madin-Darby canine kidney (MDCK) cells [106] or A431epithelial cells [107]. Therefore, EGFR may convey various downstream signaling pathways to disassemble the apical junction of polarized

epithelial cells, which eliminates the cell proliferation blockage mediated by cell-cell contact.

ErbB family receptors have also been reported to regulate the apical junction by directly interacting with polarity complexes. *Aranda et al*. have shown that activation of ErbB2 disrupts the polarity of epithelial cells by directly interacting with Par6, a component of the polarity complex that controls the polarized distribution of the actin cytoskeleton and apical localization of the tight junction. Over expressed ErbB2 leads to the redistribution of the junctional proteins, ZO-1 and occludin [108]. EGFR can regulate tight junction assembly by activating Src- and Yes-mediated phosphorylation of the polarity complex protein Par3 [109]. These studies collectively demonstrate that EGFR and ErbB family receptors can modulate the apical junction directly by interacting with the polarity complex proteins and indirectly by activating actin remodeling and proteins coupling actin to the apical junction through their downstream signaling molecules.

EGFR has also been shown to regulate the actin ctyoskeleton. Upon EGF stimulation, actin filaments accumulated at the leading edge of metastatic mammary adenocarcinoma (MTLn31) cells [110]. EGF stimulated A431 cells showed actin polymerization under EGFR [111]. EGFR-mediated actin remodeling has been shown to involve the activation of PLCγ and its products, IP3 and DAG [112]. IP_3 induces Ca^{2+} flux. The cytoplasmic Ca^{2+} and DAG activate protein kinase C (PKC). IP3 and PKC both have been reported to modulate actin rearrangement required for cell

motility [113, 114]. The inhibition of PLCγ activity blocks EGF-induced cell motility [115]. PLCγ is likely the main signaling molecule downstream EGFR that activates actin reorganization and acts via Ca^{2+} flux or PKC. Like discussed earlier, the elevated cytoplasmic Ca^{2+} activates MLCK via calmodulin, which leads to the phosphorylation and activation of NMII.

Rho-family GTPases are another signaling pathway downstream of EGFR that can regulate the actin cytoskeleton and NMII. Rho-family GTPases regulate actin by activating actin-binding proteins such as Wiskott–Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous protein (WAVE) family proteins on actin polymerization [116] and cofilin on actin depolymerization [117]. *Gohla et al*. found that the EGFR kinase inhibitor AG1478 blocks lysophosphatidic acid-induced the activation of Rho GTPase [118]. *Malliri et al*. showed that EGF stimulation induces cortical actin polymerization and membrane ruffling in a Rho- and Rac-dependent manner [111].

Rho-GTPase in turn activatesRho GTPase-associated Kinase (ROCK), which can also directly phosphorylate MLC and inhibition of NMII phosphatase. However, the mechanism by which EGFR induces the activation of Rho GTPase is poorly understood.

In summary, EGFR activation can disrupt the apical junction by inducing MLCK and/or ROCK activation, which disrupts the apical junction by inducing the reorganization of perijunctional actomyosin structures. However, the relationship between GC-induced EGFR transactivation and NMII which is associated with perijunctional actin cytoskeleton in gonococcal infected polarized epithelial cells has not been studied.

Rationale

This study was designed to gain a better understanding of the mechanism by which GC establish infection at the epithelial surface of the female reproductive tract. The goal of this study is to address the question of how GC-induced EGFR transactivation leads to the disassembly of the apical junction and whether GC interact with non-polarized and polarized epithelial cells on the endo- and exocervix differently.

Women bear the brunt of the serious sequelae of GC infections. Since female infections tend to be asymptomatic, infection often becomes chronic leading to serious complications, including DGI, PID and infertility. About 50% of infected women are under age 24, and of childbearing ages. Therefore, my Ph.D. thesis project has focused on understanding the pathogenesis of gonorrhea in women. As GC is a human exclusive pathogen, there is no adequate animal model for the disease. This has limited the research to cancer and immortalized cell lines and human tissues as models for the infection. Since the female lower reproductive tract primarily consists of both polarized and non-polarized epithelial cells, I investigated the differential interactions of GC with polarized and non-polarized of two cell lines: the human epithelial endometrial adenocarcinoma cell line HEC-1-B and the human colon epidermal carcinoma cell line T84. Both of these cell lines have been used extensively in the *N. gonorrhoeae* research. To confirm the data generated from cell lines, I established human cervix tissue explants, including endocervix lining with non-polarized squamous epithelial cells and ectocervix with polarized columnar

epithelial cells, as a model. Opa phase variation has been shown to play a role in gonococcal infection in the female reproductive tract. To examine the role of Opa in GC infection, I compared MS11mkc, a pathogenic strain that express phase variable Opa, pili and LOS containing lacto-N-neotetraose with a mutated strain where all opa genes are deleted.

Previous studies from our lab have shown that gonococcal interaction with epithelial cells induces transactivation of EGFR, and that EGFR activation is required for gonococcus-induced apical junction disruption and gonococcal transmigration across polarized epithelial cells, as well as gonococcal invasion into non-polarized cells [50, 51]. However, how EGFR signaling facilitates gonococcal transmigration and whether GC induces similar actin rearrangement in polarized and non-polarized epithelial cells for gonococcal invasion and transmigration are not known.

Central Hypothesis

Transactivation of EGFR by GC induces Ca^{2+} flux, which in turn activates MLCK subsequently NMII. Activated NMII mediates perijunctional actomyosin ring contraction, which causes the disassembly of the apical junction and promotes gonococcal transmigration across polarized epithelial cells. GC induces different actin remodeling in polarized and non-polarized epithelial cells due to different actin structures in the two types of epithelial cells, consequently resulting in distinct infection mechanisms.

Aim 1

To determine how gonococcus-induced EGFR and Ca^{2+} signaling leads to the disruption of the apical junction of polarized epithelial cells.

Aim 2

To compare the actin remodeling responses of polarized and non-polarized epithelial cells to gonococcal infection.

Chapter 2: *Neisseria gonorrhoeae* breaches the epithelial barrier by inducing Ca^{2+}-dependent activation of non-muscle myosin II

2.1 Abstract

Neisseria gonorrhoeae (GC) is the causal agent of gonorrhea in both men and women. The majority of infections in women are asymptomatic, which can lead to disseminated infection (DGI) and pelvic inflammatory diseases (PID), a common cause of infertility and ectopic pregnancy [1]. GC is known to be capable of adhering onto epithelium and invading into subepithelial tissue [31]. However, molecular and cellular mechanisms by which GC establishes infection in the female reproductive tract (FRT) remain elusive due to its human exclusive niche. The epithelium is the first-line defense of the mucosal surface with its apical junction blocking free diffusion of pathogen though the paracellular space (gate function) and prevents the later diffusion of membrane lipids and proteins (fence function). The apical junction consists of multiple integral and membrane-associated proteins. Associated protein ZO-1 and β-catenin link the apical junction complex to the actin cytoskeleton and it's signaling network in the cytoplasm. The actin motor non-muscle myosin II (NMII) is associated with actin and forms a supporting actomyosin ring in the cytoplasmic side of the apical junction, thus regulating the junction. Our recent published data demonstrate that GC interaction with polarized epithelial cells weakens the apical junction and allows them to transmigrate through in an EGFR-dependent manner

[51]. However, the molecular pathway by which GC-induced signaling leads to the apical junction disassembly remains undefined. Moreover, our preliminary data showed that the expression of GC surface molecule opacity protein (Opa) reduces the kinetics and efficacy of GC transmigration across polarized epithelial cells, which suggested these phase-varying surface molecules renders GC the capability of generating different pathological conditions of the infection. However, the molecular pathway by which Opa related signaling leads to the apical junction disassembly remains unknown.

This study examined the signaling mechanism underlying the GC-mediated junction disruption using both polarized human epithelial cells and human cervical tissue explants. Our results reveal that GC induces junction disassembly by inducing Ca^{2+} flux and the activation and redistribution of NMII. GC-induced EGFR activation leads to Ca^{2+} flux and Ca^{2+}-dependent activation of NMII, both of which are required for triggering the disassociation and endocytosis of the functional protein E-cadherin and the breakdown of both the gate and fence functions of the apical junction. Opa expression causes reductions in the levels of NMII activation and junctional disruption induced by GC as well as the morphological changes at the apical surface of epithelial cells interacting with GC. These data define the molecular mechanism by which GC takes advantage of host signal apparatus to overcome the epithelial barrier for infection.

2.2 Materials and Methods

Neisseria Strains

N. gonorrhoeae strain MS11 that expressed both pili and Opa (Pil+ Opa+) and the MS11 opa deletion mutant (Δopa) were used [12]. GC were grown on GC media base plates with 1% Kellogg's supplement (GCK) for 16–18 hr before inoculation [119]. Pil+ 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 was inoculated with epithelial cells at MOI 10:1.

Epithelial Cells

Human endometrial adenocarcinoma cell line, HEC-1-B cells (ATCC# HTB-113, Manassas VA, USA), were maintained in Eagles MEMα 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% CO_2. Cells were seeded at $6x10^4$ (6.5 mm diameter transwell) or at $1x10^5$ (24 mm diameter transwell) per transwell (3 μm pore size, polyester transwells inserts, Corning, Lowell, MA, USA) and cultured for ~10 days until transepithelial electrical resistance (TEER) reached ~400 Ω (HEC-1-B) and ~2000 Ω (T84). TER was measured using a Millicell ERS volt-ohm meter (Millipore, Bedford, MA, USA).

Human cervix transplant

Human cervix explants were obtained from National Disease Research Interchange (NDRI, PA, USA). Basically, 1/4 of cervix tissue was obtained from patients undergoing hysterectomy less than 24 hr. Cervix explants were then trimmed to get rid of most muscle tissue leaving the endocervix and ectocervix epithelium. Each piece was about 2.5 cm (L) X 0.6 cm (W) X 0.3 cm (H). These pieces were then incubated in CMRL-1066 (GIBCO, Grand Island, NY, USA) for 24 hr and switched to non-antibiotic media for experiment. The cut tissue was incubated with/without GC at MOI:10 for 24 hr with rinsing at time point 6 hr and 12 hr. The tissue was fixed and embedded in gelatin for cryosectioning. Sections were immunostained with desired antibodies for visualizing targeted proteins for their distribution.

Immunofluorescence analysis

Cells were pre-treated with or without NMII kinase inhibitors Y27632 and ML-7 (10 μM, Calbiochem, San Diego, CA, USA) or 2APB - inhibitor for IP3-induced calcium influx (Millipore, Temecula, CA, USA) and BAPTA (50 μM, Calbiochem, San Diego, CA, USA) for 1 h, and incubated with GC in the presence or absence of the inhibitors for 6 h. Cell were washed and fixed with 4% paraformaldehyde, permeabilized, and stained with anti-E-cadherin (BD Bioscience, Bedford, MA, USA), Phalloidin (Life Technology, Grand Island, NY, USA), anti-pMLC (Cell Signaling Technology, Danvers, MA, USA), and anti-GC [120] 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 0.37 μm slices from the top to the bottom of cells, and three-dimensional (3D)

composites obtained. Fluorescence intensity profiles were generated using the ImageJ software.

Immunoblotting analysis

Polarized epithelial cells apically incubated with bacteria for 6 hr in the presence or absence of inhibitors were washed with ice-cold PBS and lysed in 75 µl RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1X proteinase inhibitor cocktail (Sigma, St. Louis, MO). Cell lysates were mixed 1:1 with denaturing loading buffer. Lysates were separated through SDS-polyacrylamide gels (Bio- Rad, Hercules, CA), analyzed by western blot, and visualized using Western Lightning chemiluminescence substrate (Perkin Elmer, Boston, MA). Images were acquired and digitized directly using Fujifilm LAS-3000 CCD camera (Valhalla, NY) or acquired with x-ray film. Blots were stripped with Restore western blot stripping solution (Pierce, Rockford, IL) and re-probed with anti-β-tubulin antibody. The blots were quantified by pixel density using ImageJ software.

GC adherence, invasion and transmigration assays

Polarized epithelia cells were incubated apically with GC at 37°C for 3 hr for adherence assay and 6 hr for invasion and transmigration assays. When NMII inhibitors were used, cells were pretreated with Y27632 (10 µM), ML-7 (10 µM) or 2APB (10 µM) for 1 hr and incubated with GC in the presence of the inhibitor. For

adherence, cell-associated bacteria that were resistant to gentamicin treatment were counted as adherent GC. For invasion and transmigration, media from the basal compartment was collected and plated onto GCK to determine the number of transmigrated bacteria. Cell-associated bacteria that were resistant to gentamicin treatment were counted as invaded GC.

Ultrastructure analysis

Polarized epithelial cells were apically incubated with bacteria for 6 hr in the presence of GC. The polarized epithelial monolayer on the transwell inserts were fixed in glutaraldehyde and OsO_4 and then transferred to distilled water. The cells were dehydrated in a graded ethanol series and infiltrated with EPON 812 resin (Araldite/Medcast; Ted Pella, Redding, Ca.). After 3 hr of infiltration in whole resin, the transwell membranes were placed in flat silicone molds (Polyscience, Inc. Warrington, PA) and polymerized overnight at 60°C. Cross sections of the cells were obained by sectioning through the embedded transwell membranes. Sections were placed on bare 150-mesh copper grids, stained with uranyl acetate and lead citrate, and viewed with a ZEISS 10CA electron microscope (ZEISS, Thornwood, NY)

Statistical analysis

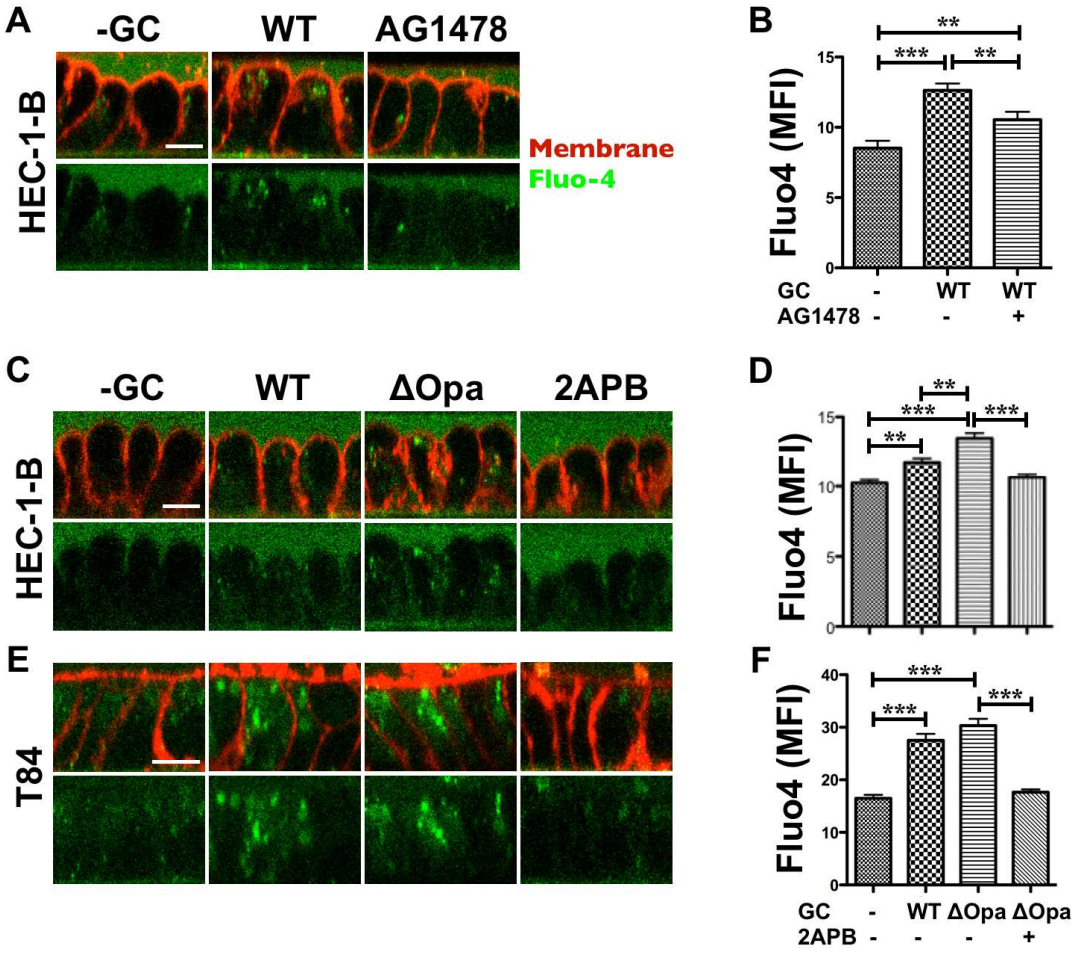
Statistical significance was assessed using the Student's t-test by Prism software (GraphPad Software, San Diego, CA). P-values were determined in comparison with controls.

2.3 Results

2.3.1 GC inoculation induces a signal-dependent elevation of the cytoplasmic Ca^{2+} in polarized epithelial cells

To investigate whether GC-induced EGFR activation leads to Ca^{2+} flux in polarized epithelial cells, I compared the levels of the cytoplasmic Ca^{2+} in the presence and absence of apically inoculated GC, the EGFR kinase inhibitor AG1478, and an inhibitor for inositol (1,4,5) triphosphate (IP3)-triggered releasing Ca^{2+} from the intracellular pool, 2APB. Polarized human endometrial epithelial cells, HEC-1-B (Fig. 6A-D), and human colonic epithelial cells, T84 (Fig. 6E-F) were pretreated with or without the inhibitors, and then incubated apically with GC MS11 wild type (wt) or a mutated strain where all the 11 opa genes were deleted (Δopa) [12] at a MOI of 10 for 4 h to allow GC to adhere, in the presence or absence of the inhibitors. The cells were then loaded with the fluorescent Ca^{2+} indicator Fluro4, marked by the fluorescent membrane dye CellMask, and imaged using three-dimensional (3D) confocal fluorescence microscopy (Fig. 6A, 6C, and 6E). The mean fluorescence intensity (MFI) of Fluo-4 in individual cells was measured to estimate the cytoplasmic level of Ca^{2+} (Fig. 6B, 6D, and 6F). Compared to cells without bacterial inoculation, polarized HEC-1-B and T84, inoculated with either wt or Δopa GC, exhibited significant increases in the MFI of Fluo-4, and Δopa GC-infected HEC-1-B cells displayed a significantly higher level of cytoplasmic Ca^{2+} than wt GC-infected cells (Fig. 6). Treatment of the EGFR kinase inhibitor partially reduced the Fluo-4 MFI (Fig. 6A-B), and treatment of 2APB brought the MFI of Fluo-4 in wt or Δopa GC-inoculated polarized epithelial cells back to the basal level as seen in

uninoculated cells (Fig. 6C-F). These results indicate that GC interacting with the apical surface of polarized epithelial cells increases the cytoplasmic level of Ca^{2+} in a signaling-dependent manner, and Opa expression is not required for but may interfere with elevating the cytoplasmic Ca^{2+} level.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ΔOpa
2APB
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Figure 6. GC inoculation induces the elevation of Ca^{2+} levels in the cytoplasm of polarized epithelial cells in an EGFR-dependent manner.

Polarized HEC-1-B cells (**A-D**) and T84 cells (**E-F**) were incubated apically with or without GC (WT or Δopa) in the presence of the Ca2+ flux inhibitor 2APB for 4 h. Then, cells were incubated with the calcium indicator Fluo-4 (green) and the membrane dye CellMask (red) and analyzed using 3D confocal microscopy. The mean fluorescence intensity (MFI) of Fluo-4 at cytoplasmic region of individual cells was measured.

(**A, C,** and **E**) Representative images. Scale bar, 5 μm.

(**B, D,** and **F**) The average MFI (±SD) of Fluo-4 staining in >50 individual cells from three independent experiments. ***p ≤0.001; **p ≤ 0.01; *p≤0.05.

2.3.2 GC interactions induce the redistribution and activation of non-muscle myosin II in polarized epithelial cells

To investigate the signaling mechanism by which GC breaches the epithelial barrier, I evaluated the impact of GC inoculation on the distribution and activation of non-muscle myosin II (NMII), a key component of the actomyosin ring associated with the apical junction [70]. As NMII-mediated contraction of the perijunctional actomyosin ring destabilizes the apical junction [72, 76, 83], I examined the cellular distribution of active NMII using 3D immunofluorescence microscopy. Active NMII was detected by antibody specific for phosphorylated myosin light chain (pMLC) that activates the ATPase activity of NMII [76]. The polarized distribution pMLC at the apical junction was quantified by fluorescence intensity ratios (FIRs) of pMLC at the junction to non-junctional (junction:nonjunction) areas of the apical surface in individual cells using confocal images scanning through the apical junction (Fig. 7A-B and 7E-F). The polarized distribution at the apical surface was quantified by FIRs of the apical to the lateral (apical:lateral) areas using images scanning across the apical and basolateral surfaces (Fig. 7C-D and 7G-H). Polarized epithelial cells incubated without bacteria and with enteropathogenic *Escherichia coli* (EPEC) served as negative and positive controls. The apical inoculation of wt and Δopa GC, but not EPEC, caused significant increases in the junction:nonjunction FIR in polarized T84 cells, compared to cells without GC inoculation (Fig. 7A-B). In contrast, the apical inoculation of wt or Δopa GC or EPEC on polarized HEC-1-B cells all led to similar levels of reduction in the junction:nonjunction FIR (Fig. 7E-F). There were significant increases in apical:lateral FIRs in both polarized T84 and HEC-1-B cells

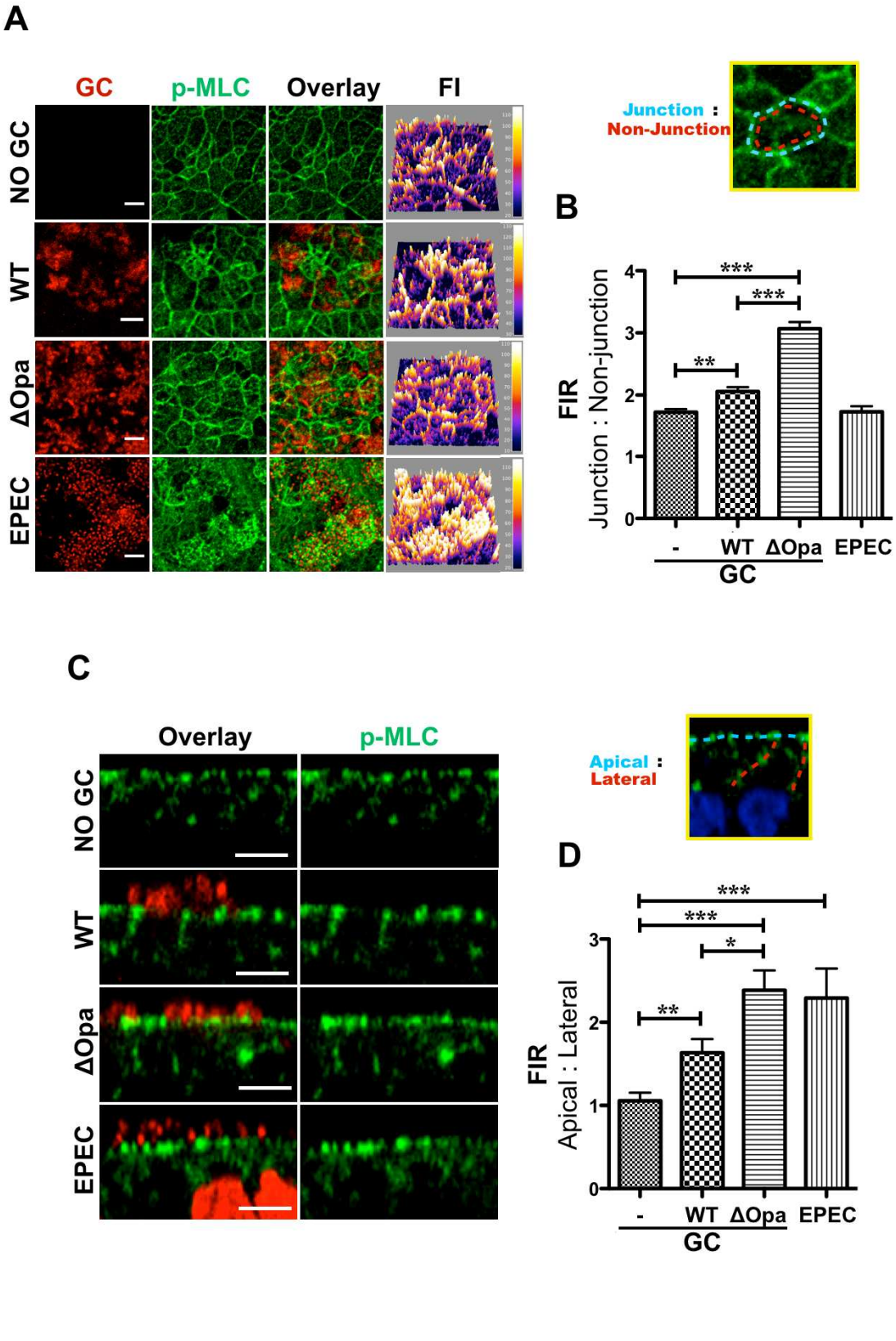
inoculated with either wt or Δopa GC, compared to the no GC control (Fig. 7E-F and 7G-H). I also noticed that the magnitude of the increases in both the junction:nonjunction FIR and the apical:lateral FIR was greater in T84 cells inoculated with Δopa GC than those in T84 cells inoculated with wt GC (Fig. 7A-D). Thus, both wt and Δopa can induce the redistribution of pMLC, increasing the levels of pMLC at the apical surface.

To determine whether GC inoculation changes the activation level of NMII, I quantified the MLC and pMLC using western blotting. Polarized T84 cells were incubated with or without wt or Δopa GC or EPEC in the apical chamber for 6 hr before lysing and western blotting. The staining density ratios of antibody specific for pMLC to MLC in Δopa GC- and EPEC-inoculated epithelial cells, but not in wt GC-inoculated epithelial cells, were significantly higher than that in uninoculated epithelial cells (Fig. 7I-J). However, none of the bacteria significantly changed the staining density ratio of MLC to tubulin (loading control) (Fig. 7K). Thus, the apical inoculation of Δopa GC, but not wt GC, increases the activation level of NMII.

To explore whether GC-induced NMII redistribution occurs *in vivo*, I utilized human endocervical tissue explants established based on previously published methods [121]. The tissue explants were incubated with wt GC at a MOI of 10 for 24 h with the removal of unattached bacteria at 6 h. Cryosections of the endocervical tissue were stained for F-actin to mark the apical surface. As expected, F-actin staining was preferentially localized at the apical surface (Fig. 8A), indicating highly polarized

endocervical columnar epithelial cells. Similar to what I observed in polarized T84 and HEC-1-B cells, there was an increased accumulation of pMLC at the apical surfaces, quantified by higher apical:lateral FIRs in GC-inoculated tissue explants, compared to no GC control (Fig. 8A-B).

Taken together, these results indicate that GC interactions increase the accumulation of activated NMII at the apical membrane area of human polarized epithelial cells in cell lines and in endocervical tissue explants. GC lacking the expression of Opa are more potent in manipulating the activity of NMII.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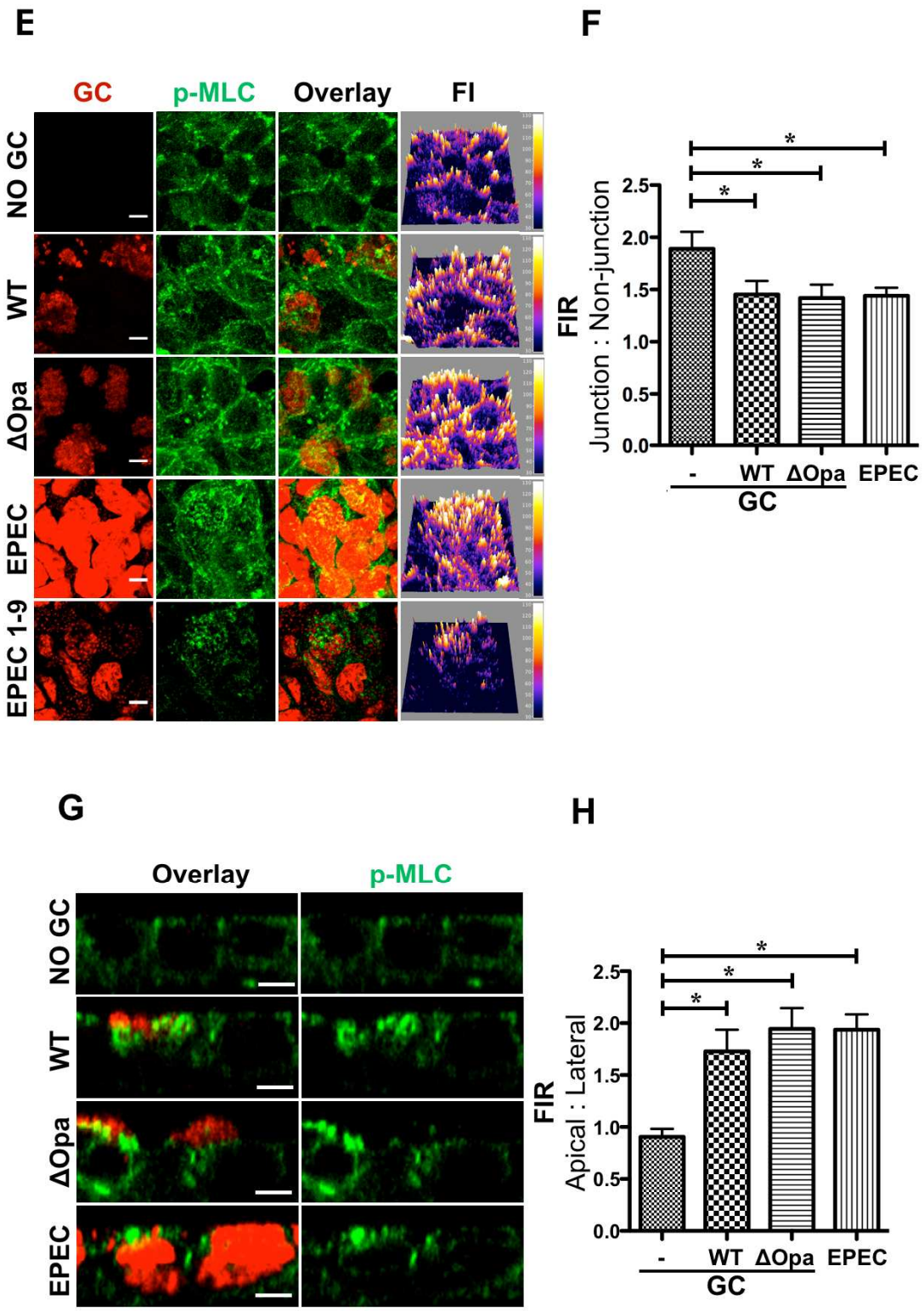
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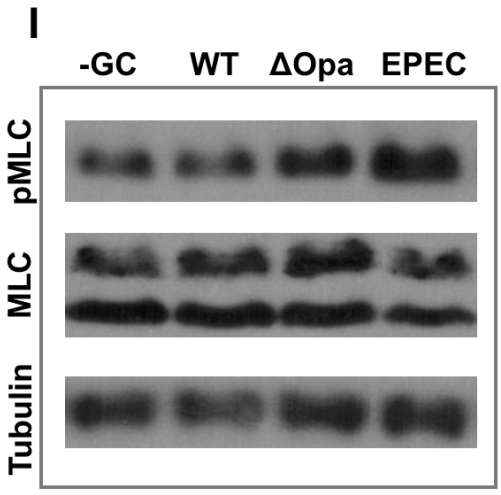
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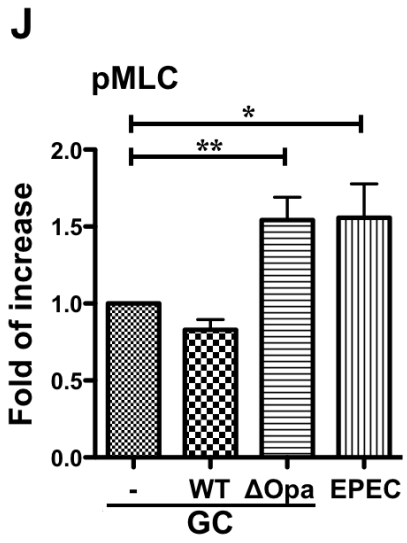
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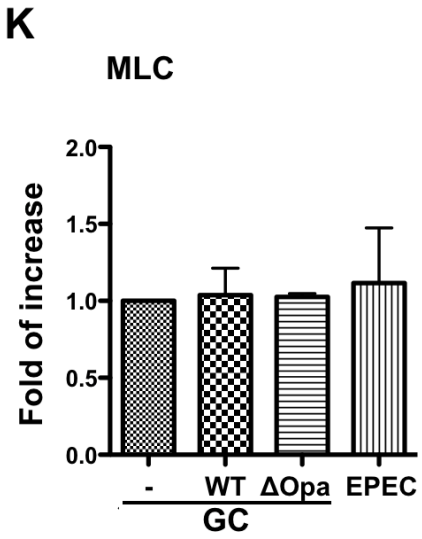
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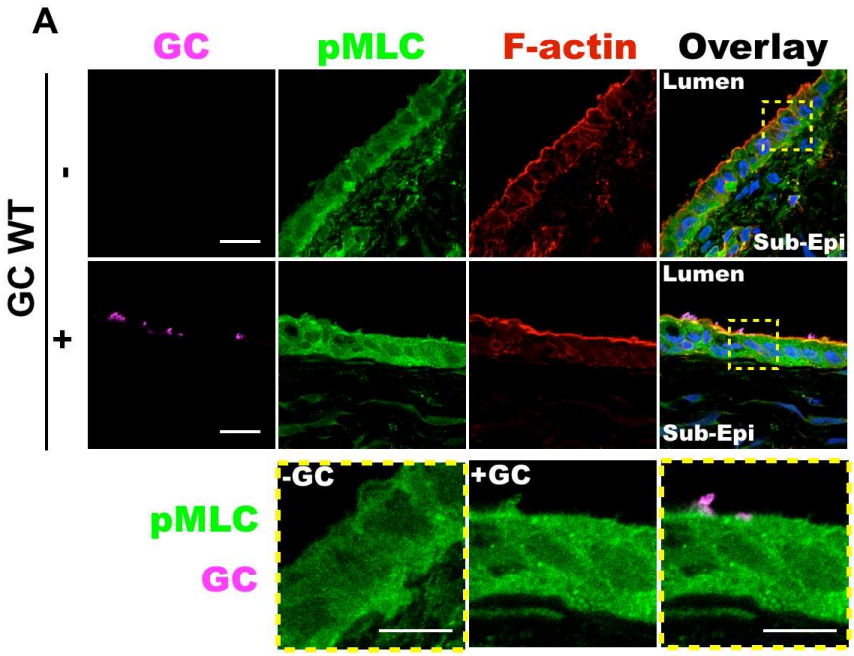
GC

Figure 7. GC interaction increases the recruitment of phosphorylated MLC to the apical junction and membrane as well as the phosphorylation level of MLC.

(**A-H**) Polarized T84 (**A-D** and **I-K**) and HEC-1-B cells (**E-H**) were incubated apically with or without GC or GC (WT or Δopa) or EPEC for 6 h. Cells were stained for phosphorylated MLC (pMLC, green) and GC (red) and analyzed using confocal microscopy. Fluorescence intensity ratios (FIR) of pMLC at the junctional to non-junctional (**B** and **F**) and the apical to lateral regions (**D** and **H**) were determined. Shown are representative xy (**A** and **E**) and xz (**C** and **G**) images, fluorescence intensity maps, and the average FIR (±SD) (**B, D, F,** and **H**) of >50 individual cells from more than three independent experiments. Scale bar, 5 μm.
(**J-K**). Polarized T84 cells were incubated apically with or without GC (WT or Δopa) or EPEC for 6 h, lysed and analyzed by SDS-PAGE and western blot probing for MLC, phosphorylated MLC (pMLC) and β-tubulin. The blot was quantified by densitometry to determine the fold increase over no GC control. Shown are represents blots (I) and the average fold of increase (±SD) (I-J) from three independent experiments.
***p ≤0.001; **p ≤ 0.01; *p≤0.05.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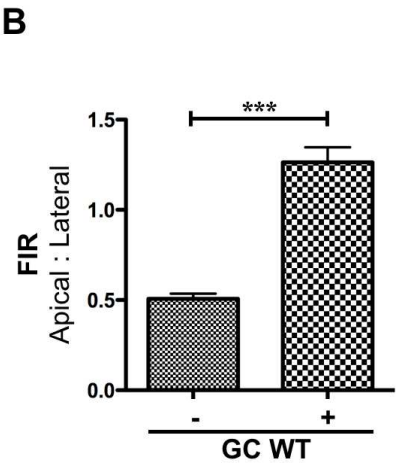
B
***
1.5
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Apical : Lateral
-
+
GC WT

Figure 8. The redistribution of active NMII in GC-infected endocervical epithelial cells of human tissue explants.

Human endocervical tissue slides were incubated with wt or Δopa GC (1x106/ml) for 6 h, washed to remove non-adherent bacteria, and continued the incubation for another 18 h. The tissue was fixed, cryopreserved, sectioned, stained for GC, pMLC, and F-actin, and analyzed using 3D confocal fluorescence microscopy.

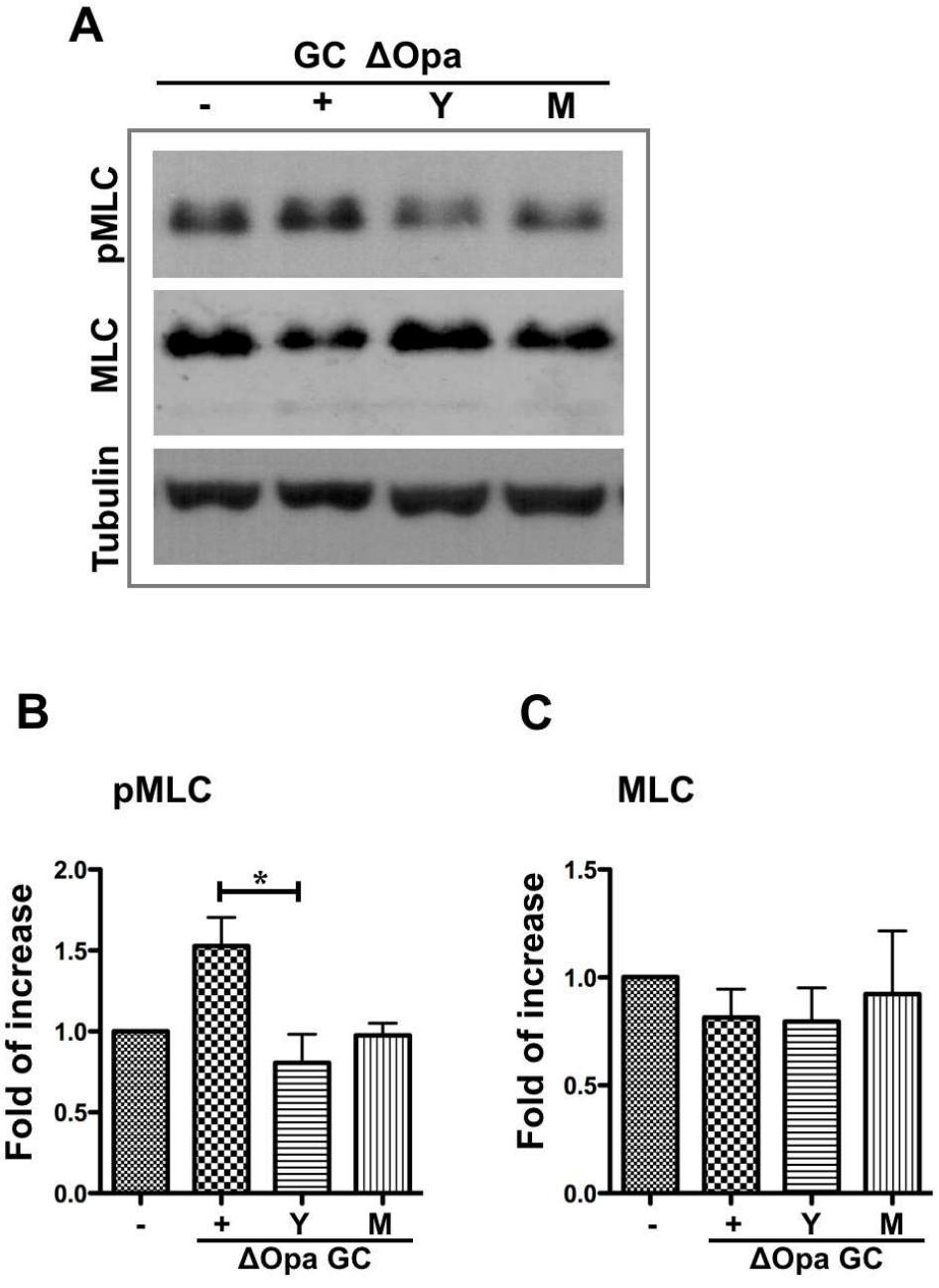
(**A**) Shown are representative images of the endocervical tissue explants stained for GC (magenta) with pMLC (green). (Scale bar - Top, 20 μm. Bottom, 10 μm.

(**B**) Fluorescence intensity ratios (FIR) (±SD) of pMLC at the apical to lateral regions in individual epithelial cells were determined. Shown are the average FIR of pMLC staining in >40 individual cells from cervixes of three human subjects. ***p ≤0.001; **p ≤ 0.01; *p≤0.05.

2.3.3 Roles of Ca^{2+}, MLCK and ROCK in GC-induced NMII activation and redistribution

The activation of NMII is generally mediated by the phosphorylation of MLC by MLC kinase (MLCK) via Ca^{2+}-activated calmodulin [74, 81, 122, 123] or by Rho-associated protein kinase (ROCK) via Rho family GTPases [74, 78]. To examine how GC regulates NMII activity, I determined the impact of MLCK, ROCK and Ca^{2+} inhibitor on GC-induced MLC phosphorylation and redistribution using western blotting and 3D immunofluorescence microscopy. Our western blot analysis showed that treatment of either the MLCK or the ROCK inhibitors reduced the pMLC:MLC density ratio, but not the MLC:tubulin density ratio, in Δopa GC-inoculated epithelial cells compared to the basal levels as seen in non-inoculated epithelial cells (Fig. 9A-C). This result suggests that both MLCK and ROCK are involved in Δopa GC-induced MLC phosphorylation. Our 3D immunofluorescence microscopic analysis found that treatment of the MLCK inhibitor ML-7 reduced both the junction:nonjunction and apical:lateral FIRs of pMLC in GC-inoculated epithelial cells to the basal level (Fig. 9D-E). However, treatment with the ROCK inhibitor Y27632 further increased the junction:nonjunction FIR of pMLC in Δopa GC-inoculated epithelial cells, while having similar inhibitory effects as the MLCK inhibitor on the apical:lateral FIR of pMLC (Fig. 9D-F). Treating polarized epithelial cells with 2APB, which inhibits Ca^{2+} release from the intracellular pool, or the Ca^{2+} chelator BAPTA also significantly decreased the junction:nonjunction FIR of pMLC to the basal level (Fig. 9G-H). These results suggest that both MLCK and ROCK

contribute to GC-induced NMII activation and accumulation of activated NMII at the apical region; however, Ca^{2+} and downstream MLCK promote the recruitment of active NMII to the apical junction while ROCK inhibits this process.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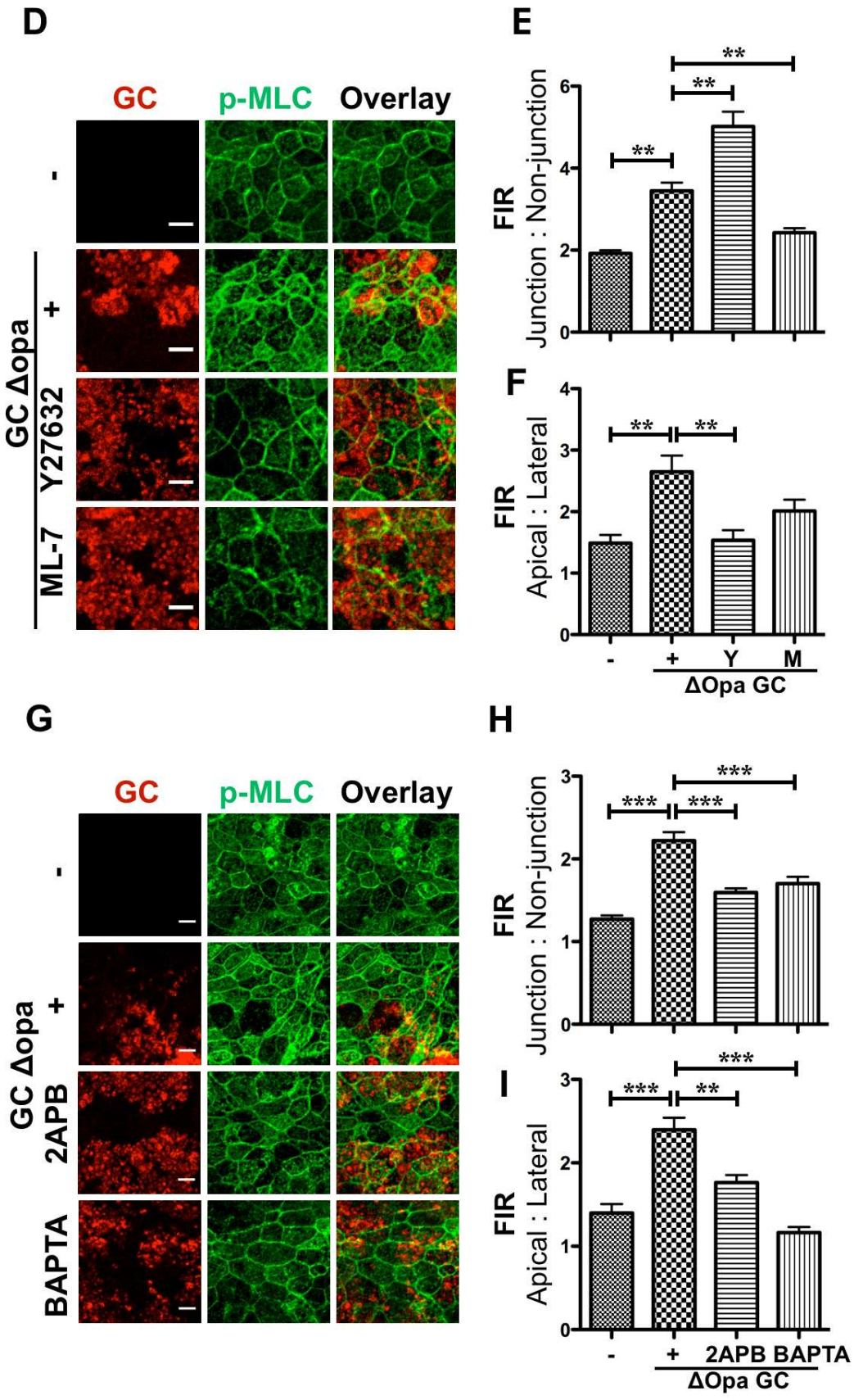
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ΔOpa GC

Figure 9. GC-induced NMII redistribution depends on NMII phosphorylation and Ca^{2+} flux.

Polarized T84 cells were untreated or pre-treated with the ROCK inhibitor Y27632 (10 μM) (**A-F**), MLCK inhibitor ML-7 (10 μM) (**A-F**), the calcium inhibitor 2APB (10 μM) or calcium chelator BAPTA (50 μM) (**G-I**) for 1 h, and then apically incubated with GC Δopa for 6 h in the presence or absence of inhibitors.

(**A-C**) Cells were lysed and analyzed by SDS-PAGE and Western blot probing for MLC, pMLC and β-Tubulin. The blot was quantified by densitometry. Shown are representative blots and the average fold increase (±SD) over no GC control from three independent experiments.

(**D-I**) Cells were stained for pMLC (green) and GC (red) and analyzed using confocal microscopy. The FIRs at the junctional to non-junctional and at the apical to lateral in individual cells were determined. Shown are representative images (**D** and **G**) and the average FIR (±SD) in >50 epithelial cells from three independent experiments (**E-F** and **H-I**). ***p ≤0.001; **p ≤ 0.01; *p≤0.05.

2.3.4 Ca^{2+} signal and NMII are required for GC to efficiently transmigrate across polarized epithelial cells

To investigate whether GC-induced Ca^{2+} elevation and NMII redistribution are involved in GC infection, I analyze the effect of Ca^{2+} and NMII kinase inhibitors on the ability of GC adherence onto, invasion into, and transmigration across the polarized epithelial monolayer. Our results showed that Δopa GC transmigrated from the apical surface of polarized T84 cells into the basolateral chamber in a much greater number than wt GC (Fig. 10A). The Ca^{2+} and MLCK inhibitor, but not the ROCK inhibitor, significantly reduced the transmigration of Δopa GC across polarized epithelial cells (Fig. 10A). The inhibitory effect of the Ca^{2+} inhibitor 2APB (~1,000 fold) was much more dramatic than the MLCK inhibitor ML-7 (~10 fold) (Fig. 10A). In contrast, none of these inhibitors had any significant effect on the adherence (Fig. 10B) and invasion (Fig. 10C) of Δopa GC. These results suggest that the elevated cytoplasmic Ca^{2+} and the phosphorylation of MLC by MLCK in the cytoplasm of polarized epithelial cells are required for optimal levels of GC transmigration, but not for GC adherence and invasion.

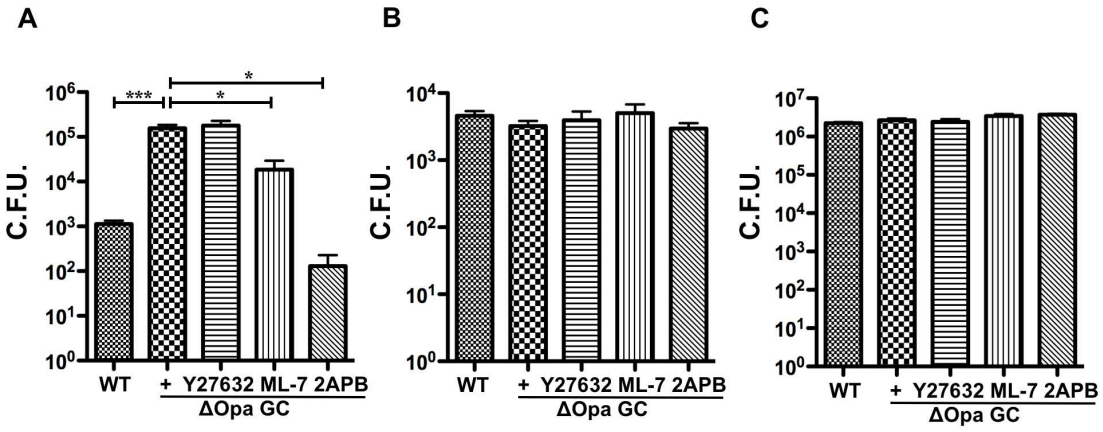


Figure 10. Inhibition of NMII phosphorylation and its upstream Ca^{2+} signal reduces GC transmigration, but not GC adherence and invasion.

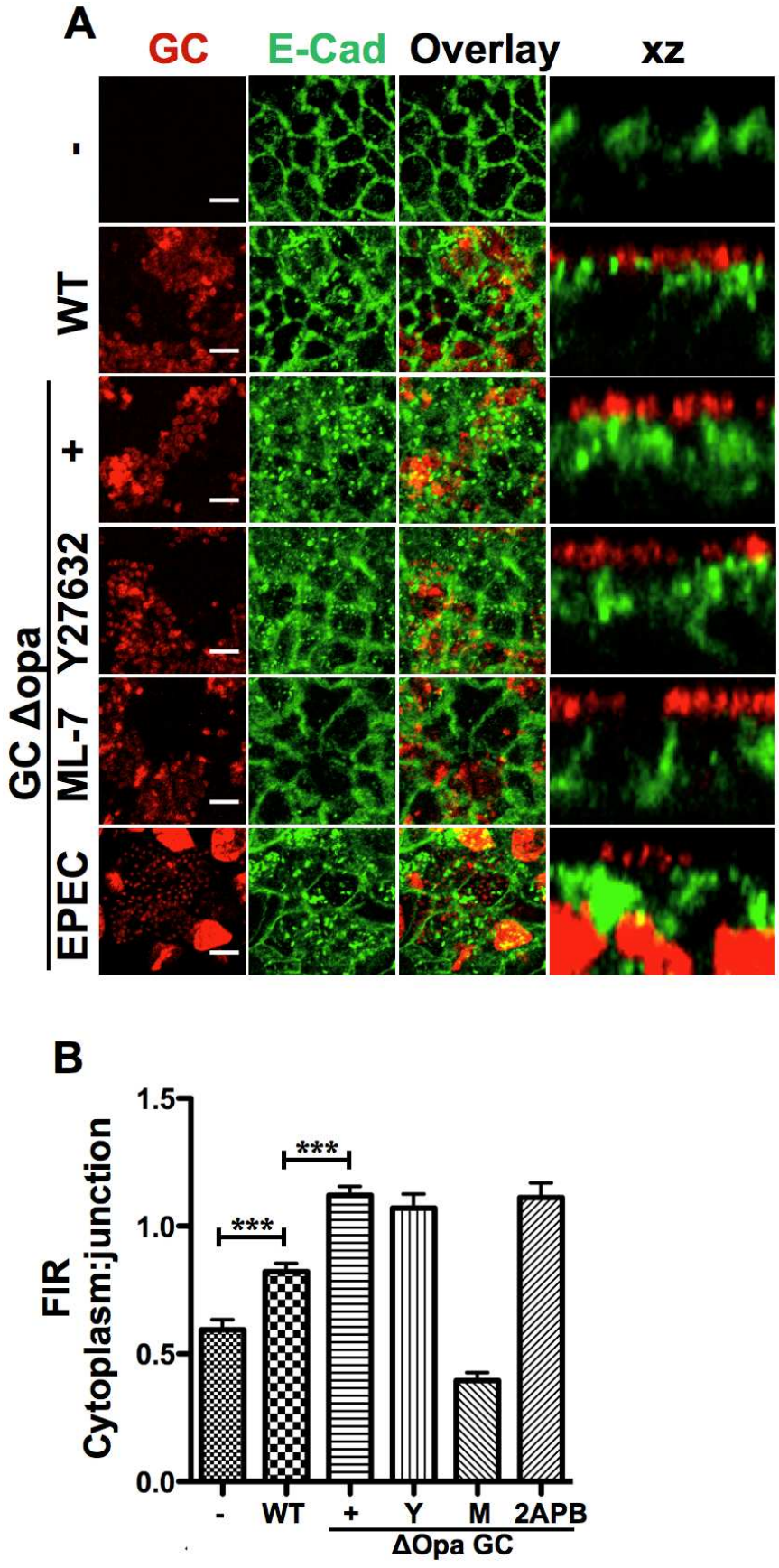
Polarized T84 cells were untreated or pre-treated with the ROCK inhibitor Y27632 (10 μM) the MLCK inhibitor ML-7 (10 μM) or the intracellular calcium release inhibitor 2APB (10 uM) for 1 h, and apically incubated with GC (Δopa) for 6 h in the presence or absence of inhibitors. The basal medium was collected to determine transmigrated GC (**A**). Invaded GC (**B**) and adhered GC (**C**) were quantified by the gentamicin resistance assay. Data shown here are the average values (± SD) of 4-6 independent experiments. *$p \leq 0.05$.

2.3.5 GC-induced junction disassembly depends on NMII activation

Our lab has previously shown that GC can disrupt the apical junction, which facilitates GC transmigration across the epithelium [124]. NMII is known to regulate the structure and function of the apical junction by mediating contraction of perijunctional actomyosin [44, 55, 72]. To determine whether NMII is involved in GC-induced disassembly of the apical junction, I analyzed how the blockage of GC-induced NMII activation and redistribution by the MLCK and Ca^{2+} inhibitors impacts the structural and functional integrity of the apical junction. The structural integrity of the apical junction was determined by analyzing the distribution of E-cadherin using 3D-immunofluorescence microscopy and by quantifying the FIR of E-cadherin at the cytoplasm to that at the apical junction. In polarized T84 cells that were not inoculated with GC, E-cadherin staining was primarily localized at the apical junction region (Fig. 11A). While the apical inoculation of wt GC did not have significant effects on E-cadherin distribution, the incubation with Δopa GC changed continuous E-cadherin staining at the apical junction into puncta in the cytoplasm, indicating the endocytosis of E-cadherin (Fig. 11A). This led to a significant increase in the cytoplasm:junction FIR of E-cadherin (Fig. 11B). Treatment of the MLCK inhibitor ML-7, but not the ROCK inhibitor Y27632, significantly decreased the cytoplasmic punctate staining and the cytoplasm:junction FIR of E-cadherin (Fig. 11A-B). Furthermore, both the Ca^{2+} inhibitor 2APB and BAPTA reduced the cytoplasmic staining of E-cadherin and brought the cytoplasm:junction FIR of E-cadherin back to the control level as seen in epithelial cells without GC inoculation (Fig. 11C-D).

The apical junction functions to control both the diffusion through the paracellular space (gate function) and the lateral diffusion between the apical and basolateral membrane (fence function), which maintains structural and functional polarity of the two membranes. To analyze the lateral diffusion between the apical and basolateral membrane, I stained the basolateral surface exclusively with the CellMask dye, which becomes fluorescent only when it is inserted into membrane, after polarized epithelial cells were incubated with Δopa GC for 6 h. The appearance of basolaterally stained CellMask dye at the apical surface indicates a decrease in the fence function. In control epithelial cells that were not inoculated with bacteria, less than 10% of cells showed the CellMask staining at the apical surface. The percentages of cells with apical staining of basolaterally labeled CellMask increased to ~20% when wt GC was inoculated, and to ~60% when Δopa GC was inoculated, indicating that Δopa GC caused a greater reduction in the fence function of the apical junction than wt GC (Fig. 12A-D). The treatment of cells with the MLCK inhibitor ML-7 or the Ca^{2+} inhibitor 2APB significantly lowered the percentage of epithelial cells with the apical CellMark staining (Fig. 12A-D), thereby inhibiting GC-induced fence function reduction. I evaluated the paracellular diffusion of the polarized epithelial cells by measuring transepithelial electric resistance (TEER). Compared to epithelial cells without incubation with bacteria, both Δopa GC and EPEC in 6 hr significantly reduced the TEER level (Fig. 12E). Treatment of the Ca^{2+} inhibitor 2APB restored TEER to the control level, but the MLCK and ROCK inhibitors failed to do so (Fig. 12E).

These data together show that elevated cytoplasmic Ca^{2+} and Ca^{2+}-dependent NMII activation are required for GC-induced disassembly of the apical junction and ablation of the barrier function of the apical junction.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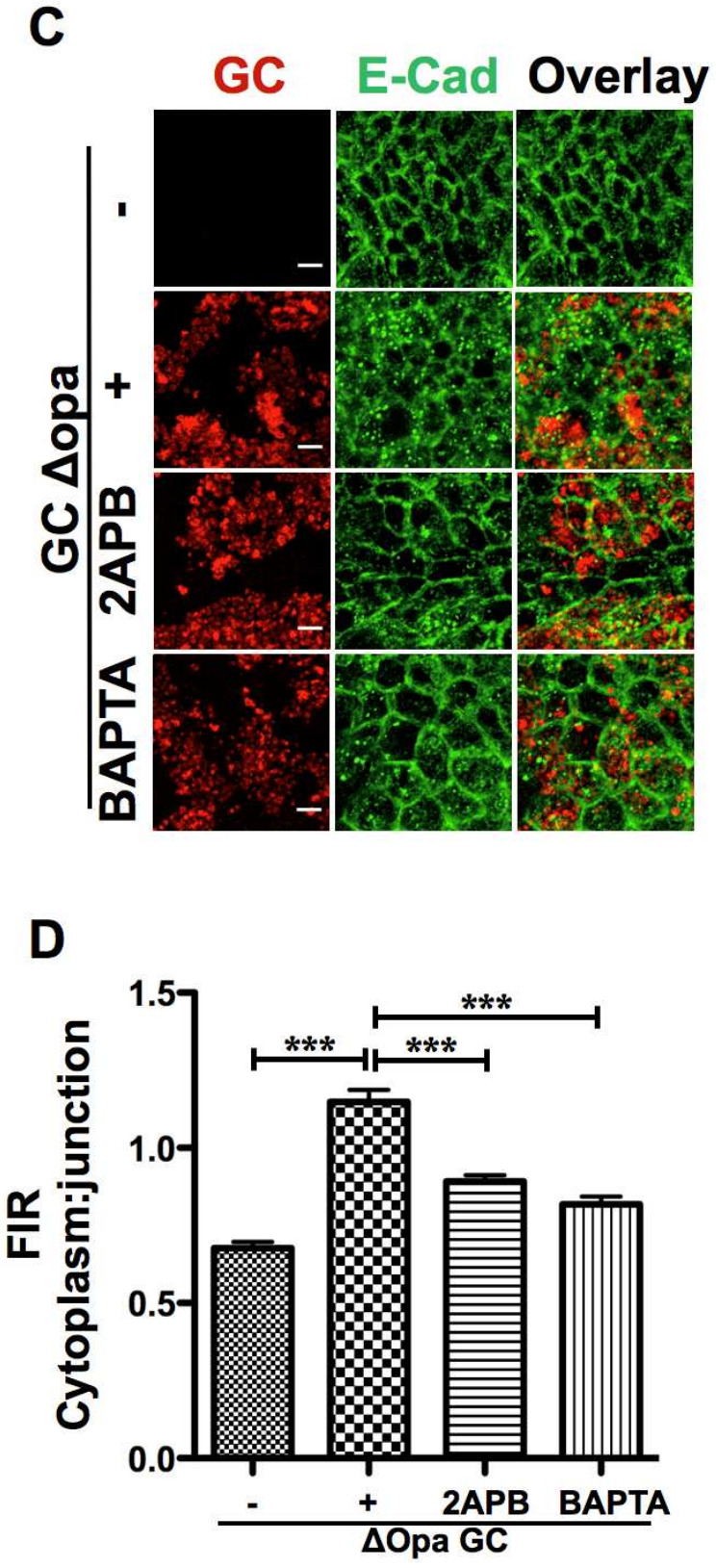
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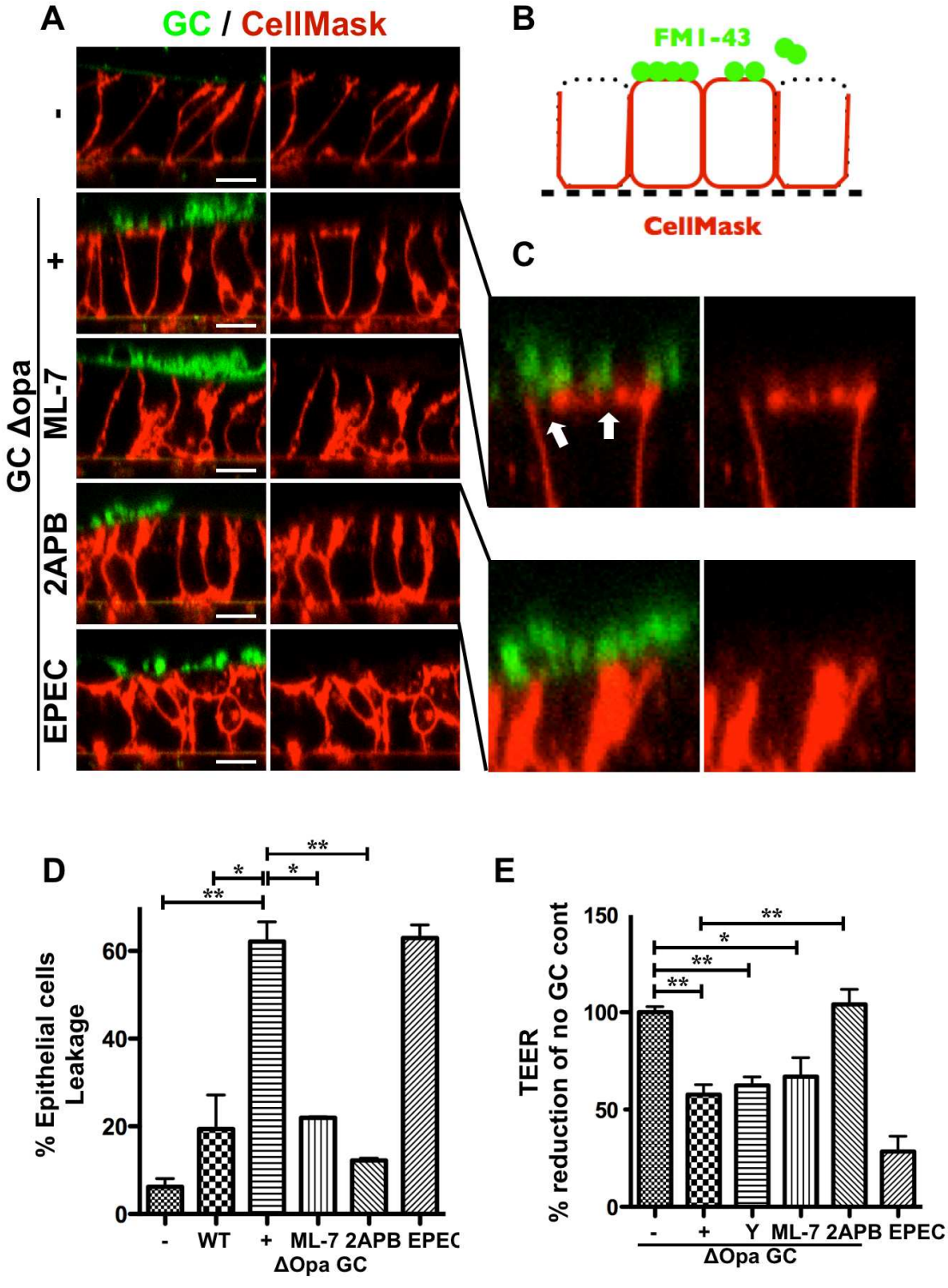
ΔOpa GC

Figure 11. GC-induced E-cadherin redistribution depends on Ca^{2+} signal and NMII activation.

Polarized T84 cells were untreated or pre-treated with NMII kinase inhibitor Y27632 (Y) and ML-7 (**A**-**B**), or Ca^{2+} inhibitor 2APB or BAPTA (**C**-**D**) for 1 h, and then apically incubated with GC ΔOpa for 6 h in the presence or absence of inhibitors. Cells were fixed, stained for E-cadherin (E-Cad) and GC, and analyzed using 3D confocal microscopy.

(**A** and **C**) Shown are representative images. Scale bar, 5 μm.

(**B** and **D**) The FIR of E-Cad staining at the cytoplasmic regions to the cell-cell junctional region was determined from images sliced through the apical junction. Shown is the average FIR (±SD) in >50 epithelial cells from three independent experiments. *** $p \leq 0.001$.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GC / CellMask
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ΔOpa GC

Figure 12. GC-mediated disruption of the barrier function of the apical junction requires Ca^{2+} signal and NMII activation.

Polarized T84 cells seeded in the bottom of transwells were untreated or pre-treated with the MLC kinase inhibitor ML-7, the ROCK inhibitor Y27632, or the intracellular calcium release inhibitor 2APB for 1 h, and then apically incubated with Δopa GC or EPEC for 6 h in the presence or absence of inhibitors.

(**A-D**) GC (green) and basolateral surface (red) were stained with different lipid dyes for 15 minutes. Time lapse images were acquired using a confocal microscope. The percentage cells with the appearance of the basolaterally stained dye in the apical surface was determined by visual inspection (**D**). Shown are representing images (**A** and **C**) and the average percentage (±SD) of cells showing basolaterally stained dyne in the apical surface from three independent experiments. Scale bar, 5 μm.

(**E**) The transepithelial electric resistance (TEER) was determined. Shown are the mean TEER values (±SD) of 10 transwells from four independent experiments.

***p ≤0.001; **p ≤ 0.01; *p≤0.05.

2.3.6 Differential interactions of wt and Δopa GC with the apical surface of polarized epithelial cells

Our finding that Δopa GC is more potent in activating NMII, disrupting the apical junction, and transmigrating across the epithelium than wt GC suggests that Opa expression may alter the interaction of GC with polarized epithelial cells. I analyzed the interaction by transmission electron microscopy (TEM). Polarized T84 cells were incubated apically with or without wt or Δopa GC at a MOI of 50 for 6 h before processing for TEM. In the absence of bacteria, the apical surface was rich in microvilli, and the dense junctional protein complex (white arrow) was readily visible at the apical region between neighboring cells (Fig. 13Aa). In epithelial cells inoculated with wt GC, microvilli were largely intact, and most of the bacteria (78%) contacted the tip of the microvilli, while a small portion of the bacteria formed broad contact with the apical surface (black arrow) (Fig. 13Ab). In contrast, most of Δopa GC (78%) formed broad and intimate interactions with the apical surface, where most of the diplococcal surface was surrounded by the host cells membrane, particularly those bacteria localizing at the top of the apical junction (red arrows) (Fig. 13Ac). The apical surface of epithelial cells interacting with Δopa GC lost most microvilli. Furthermore, the density of the apical junctional complexes in epithelial cells interacting with GC appeared to be decreased, compared to those epithelial cells without bacteria (open arrows) (Fig. 13Ab and 13Ac). Consistent with these results, our 3D immunofluorescence microscopic analysis found that wt GC colonized at the apical surface of epithelial cells was located at the top of the NMII(A) staining, while Δopa GC are partially colocalized with the NMII(A) staining (Fig. 13B). These

results indicate that phase-varying of Opa can dramatically change the way by which GC interacts with polarized epithelial cells.

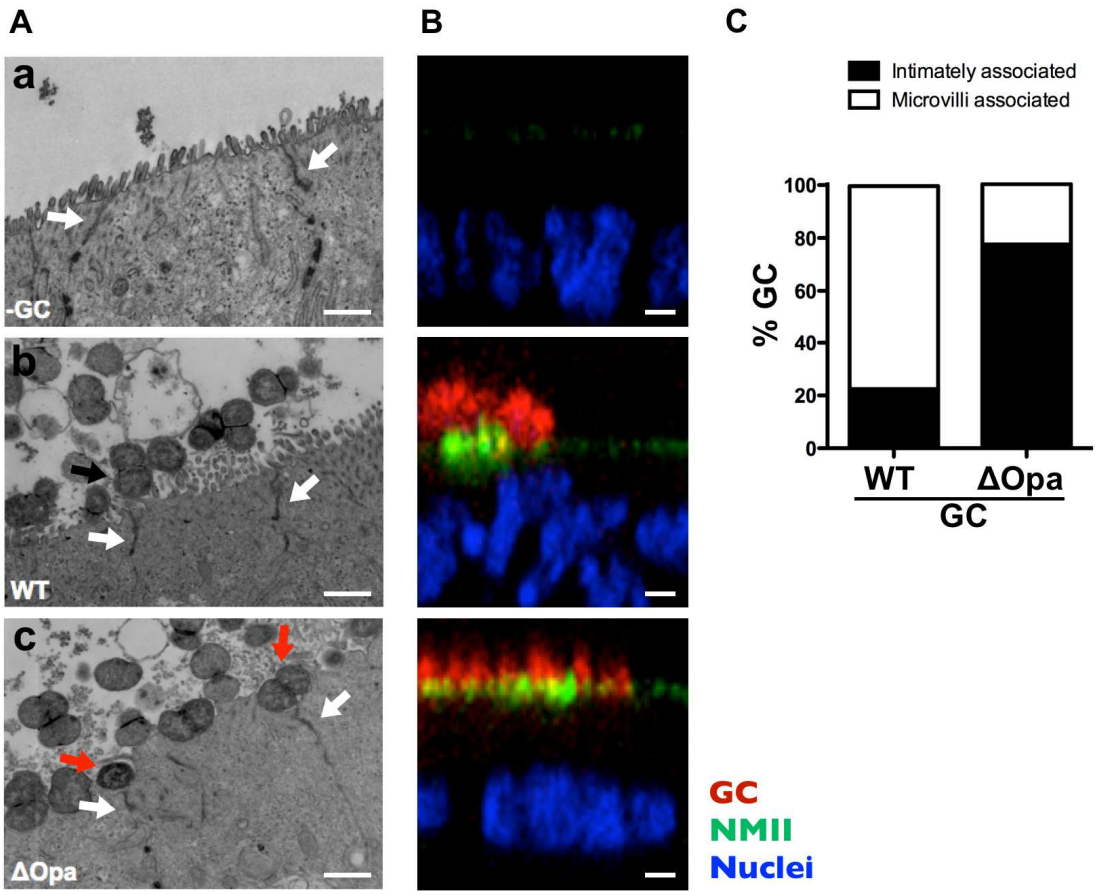


Figure 13. Opa expression alters the nature of GC interaction with the apical surface of polarized epithelial cells.

Polarized T84 cells were incubated apically with or without wt and Δopa GC for 6 h (MOI 50).

(**A** and **C**) Cells were fixed and processed for transmission electron microscopy. Images were acquired using Zeiss EM10 (**A**). The percentages of GC with extensive contact with the epithelial membrane and loose association with microvilli were determined from two independent experiments. Scale Bar, 1 μm.

(**B**) Cells were fixed, stained for GC (red), NMIIA (green) and nuclei (blue), and analyzed using confocal fluorescence microscopy. Shown are representative images from three independent experiments. Scale Bar, 2 μm.

2.4 Discussion

The results presented in this chapter demonstrate that the interaction of GC with the apical surface of the polarized epithelial cells elevates the cytoplasmic Ca^{2+} level and induces Ca^{2+}-dependent redistribution of active NMII. Both Ca^{2+} flux and NMII redistribution are required for GC-induced disruption of the apical junction, leading to depolarization of epithelial cells and efficient transmigration of GC across the epithelium. Importantly, the expression of Opa inhibits rather than enhances GC-induced elevation of cytoplasmic Ca^{2+}, activation and cellular redistribution of NMII, and junction disruption, as well as intimate interaction of GC with the apical surface of epithelial cells, consequently reducing GC transmigration.

Our previously published studies have demonstrated that the interactions of GC with polarized epithelial cells induce the EGFR transactivation and the disassembly of the apical junction in EGFR-dependent manner [124]. Alterations in the cytoplasmic Ca^{2+} level in GC-infected epithelial cells have also been reported previously [9, 97]. However, the mechanism which GC influences the cytoplasmic Ca^{2+} level of epithelial cells and the significance of the change in the cytoplasmic Ca^{2+} level in GC infection were unknown. This study reveals that cytoplasmic Ca^{2+} and NMII are downstream of EGFR and Ca^{2+} respectively, linking EGFR transactivation to the cytoplasmic Ca^{2+} and NMII activation. Based on the current literature, there are several possible mechanisms for GC to increase the cytoplasmic Ca^{2+} [9], including integration of porin onto the plasma membrane and intracellular membrane [97], membrane damage caused by pili contraction [94], and EGFR transactivation [100].

The first two mechanisms likely trigger Ca^{2+} influx from the extracellular pool independent of signaling [9], while EGFR activation induces Ca^{2+} flux via activating PLCγ. PLCγ generates IP_3, activating Ca^{2+} release from the intracellular pool via IP_3 receptor, which in turn activates Ca^{2+} influx from the extracellular pool [125-128]. Here I found that GC-induced Ca^{2+} elevation and NMII redistribution are blocked by the inhibitor for signaling-triggered release of intracellular Ca^{2+} pool and that the Ca^{2+} elevation is inhibited by the EGFR kinase inhibitor. These findings indicate that GC induces Ca^{2+} flux by triggering signal transduction, particularly the transactivation of EGFR signaling, rather than the plasma membrane injury by porin or pili retraction. However, the EGFR kinase inhibitor only partially inhibits GC-induced cytoplasmic Ca^{2+} elevation, which may be due to a relatively low concentration of the inhibitor or the involvement of other signaling pathways in GC regulation of the cytoplasmic Ca^{2+}, in addition to EGFR.

I demonstrated here that GC interaction can increase the levels of NMII activation and accumulation at the apical surface and junction region of polarized epithelial cells, and that the NMII activation and redistribution depend on the cytoplasmic Ca^{2+} elevation. Similar results from endometrial and colonic epithelial cell lines and human endocervical tissue explants suggest that GC-induced NMII activation and apical accumulation occur in vivo. The activation of NMII requires the phosphorylation of MLC, which can be mediated by two kinases, MLCK and ROCK [74]. Here I show that in Δopa GC-infected epithelial cells, while both the MLCK and ROCK inhibitors inhibit GC-induced MLC phosphorylation and NMII apical

accumulation, only the MLCK inhibitor blocks the NMII junctional accumulation. Furthermore, the MLCK inhibitor, but not the ROCK inhibitor, blocks GC-induced junctional disruption and GC transmigration. These results suggest that the activation of NMII at the apical junction is mediated by MLCK, and Ca^{2+}-calmodulin activated MLCK is required for GC-mediated junction disassembly. The inhibitory effects of Ca^{2+} inhibitors on GC-induced junctional disruption further support the notion. Our results also suggest that ROCK-activated NMII plays different roles from MLCK-activated NMII during GC infection.

Increased levels of active NMII at the apical junction are known to lead to the disruption of the apical junction [72]. This study shows that GC utilizes this mechanism to disrupt both the gate and fence function of the apical junction compromising the polarity of epithelial cells. The apical junction in individual cells is structurally supported and functional regulated by a perijunctional actomyosin ring [80, 129-131]. The actomyosin ring associates with the junctional complex via ZO-1 of the tight junction and α/β–catenin of the adherens junction [57, 132-134]. NMII-mediated contraction along perijunctional actin can transiently open the apical junction for paracellular secretion and induce the disassembly of the apical junction [135, 136]. In addition to NMII activation, I previously showed another mechanism for GC to trigger junction disruption, where GC interactions induce the phosphorylation of β-catenin, which disassociates β-catenin from the apical junction and leads to the detachment of the actomyosin ring from the junctional protein complexes. Our findings that the phosphorylation of β–catenin [124] and NMII

depends on EGFR kinase activity and EGFR-induced Ca^{2+} flux suggest that both pathways are downstream of GC-triggered EGFR transactivation.

Consistent with our previous published data [124], this study further confirms that the disruption of the apical junction induced by NMII activation and redistribution facilitates the transmigration of GC across polarized epithelial cells. The positive correlation between the apical junction disassembly and GC transmigration implies that GC invasion into the subepithelial tissue requires junction disruption and that GC can migrate across the epithelium paracellularly through compromised apical junction. Besides subepithelial invasion, disruption of the apical junction compromises the polarity of epithelial cells, consequently altering the intracellular organization and functional properties of the cells, which may provide additional benefits for GC survival and infection in the genital track. For example, basolaterally located surface receptors, such as CD46 [137], or extracellular matrix [138] become available for GC to bind and utilize for adherence and invasion, and the reorganization of the cytoskeleton in epithelial cells may transform the apically centric and structurally rigid actin bundles and rings to forms that can aide GC adherence and invasion. The polarity changes of epithelial cells may also modify their immune sensing function.

An important finding of this study is that Opa expression interferes with rather than facilitates GC-mediated junction disruption, consequently reducing GC transmigration, as GC strain that lacks all 11 Opa genes disrupts the apical junction

and transmigrates across polarized epithelial monolayers to greater magnitudes than its wt counterpart. Our mechanistic examination shows that Δopa GC, but not wt GC, causes an increase in the level of phosphorylated MLC, and Δopa GC induces the cytoplasmic Ca^{2+} elevation and NMII redistribution to greater levels than wt GC. These results confirm the link between GC-induced NMII activation and apical junction disruption. However, how Δopa and wt GC differentially regulate NMII activation is largely unknown. Our previous studies showed that the binding of Opa to the surface LOS on adjacent bacteria provides strong GC-GC interactions, leading to the formation of tight microcolonies on the surface of epithelial cells, while Δopa GC fail to form microcolonies and spread on epithelial cells [12]. Strong GC-GC interactions likely interfere with GC interaction with epithelial cells. Indeed, our electronic microscopic analysis here shows that while wt GC interacts with tips of epithelial microvilli, Δopa GC interacts with the apical membrane of polarized epithelial cells intimately and extensively, consequently abolishing most of microvilli. Differences in the physical interactions between GC and epithelial cells visualized by electron microscopy reflect different nature and extent of molecular interactions between the surfaces of GC and epithelial cells, which could lead to distinct signaling in the cytoplasmic of epithelial cells. Our previous studies found that non-polarized epithelial cells infected with phenotypic Opa-negative GC (identified based on the colony opacity) exhibited lower levels of EGFR phosphorylation than those infected with Opa-expressing GC [51], which argues against the above hypothesis. However, the effects of Opa gene deletion on GC's ability to transactivate EGFR in polarized epithelial cells is unknown and require

further examination. These results together suggest that Opa phase variation can changes the way and magnitude of GC to induce signaling and actin remodeling in epithelial cells.

While the molecular details in the infection process of GC remain to be defined, the accumulated data from previous and this study enable us to postulate the following working model. GC interactions with polarized epithelial cells, such as endocervical epithelial cells in the female reproductive tract, induce the transactivation of EGFR and the elevation of the cytoplasmic Ca^{2+}, which in turn activate the phosphorylation of β-catenin and NMII. The phosphorylation of β-catenin and NMII leads to the disassembly of the apical junction and the detachment and remodeling of the perijunctional actomyosin structure, compromising the functional polarity of epithelial cells and facilitating GC invasion into subepithelial tissues. The phase variation of Opa protein enables the bacteria to form different interactions among themselves and with polarized epithelial cells, thereby inducing signaling and actin remodeling in different manners and magnitudes, consequently differentially modifying the efficacy of GC adherence, invasion and transmigration. Therefore, Ca^{2+} flux and downstream NMII are regulatory targets of GC for varying the infection mechanisms and infection complications while changing GC surface molecules.

Chapter 3: Interaction of Neisseria gonorrhoeae with polarized and non-polarized epithelial cells at the endocervix and ectocervix

3.1 Abstract

GC infects the female reproductive tract. GC has been found in the subepithelial region, suggesting transmigration of the bacteria across the epithelium. The ability of GC to invade into the subepithelial tissue is associated with gonorrhea recurrence and inflammatory complications. GC encounters the epithelium of the lower female reproductive tract first. There are two types of epithelial cells lining the surface of the lower female reproductive tract: non-polarized multi-layer squamous epithelial cells and polarized monolayer columnar epithelial cells. The two types of cells from the same epithelial cell origin have distinct properties. Polarized epithelial cells have apical cell junctions that seal paracellular space and form a gate to separate cell membrane from the apical lumen region to basal extracellular matrix region. The apical and basal regions therefore develop with different functionality and lipid/protein compositions. On the other hand, non-polarized epithelial cells do not have or have different apical junctions and all the lipid/protein compositions are evenly distributed in/along the cell. The different cell properties between polarized and non-polarized epithelial cells makes the cell respond to pathogens and induce signaling pathways in different ways. To establish an infection, GC attaches to the luminal side of the epithelial surface, invade into epithelial cells and/or transmigrate across epithelial cells. As the squamous non-polarized epithelial layers of

ectocervixand the columnar polarized epithelial monolayer of endocervix have different cell-cell junctions and actin organization, it is not known whether GC interact with them in the same way and infect them using the same mechanism.

This study compares the interaction of GC with polarized and non-polarized epithelial cells and the ability of GC to adhere, invade, and transmigrate across polarized and non-polarized epithelial cells. It also examines GC-induced actin remodeling in polarized and non-polarized epithelial cells, using both cell lines and human cervical tissue explants as models. Our results show that T84 and HEC-1-B epithelial lines are able to mimic ectocervical and endocervical epithelium and represent cell properties and polarity under different times of growth. The actin cytoskeleton and pMLC distribution is different between polarized and non-polarized epithelial cells. In the presence of GC, actin is recruited under the GC adherent site in the non-polarized epithelial cells, but reduced in the polarized epithelial cells. pMLC is recruited to the GC adherent site in polarized epithelial cells, but remains the same in non-polarized epithelial cells. There is a correlation between actin and pMLC in polarized epithelial cells, suggesting that pMLC contributes to actin disassembly. These results suggest that the polarity of epithelial cells at different anatomic locations of the female reproductive tract alters the mechanisms by which GC establishes an infection.

3.2 Materials and Methods

Neisseria Strains

N. gonorrhoeae strain MS11 that expressed both pili and Opa (Pil+ Opa+) and the MS11 opa deletion mutant (Δopa) were used [12]. GC were grown on GC media base plates with 1% Kellogg's supplement (GCK) for 16–18 hr before inoculation [119]. Pil+ 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.

Epithelial Cells

Human endometrial adenocarcinoma cell line, HEC-1-B cells (ATCC# HTB-113, Manassas VA, USA), were maintained in Eagles MEMα 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% CO_2. Cells were seeded at $6x10^4$ (6.5 mm diameter transwell) or at $1x10^5$ (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 (TEER) reached ~400 Ω (HEC-1-B) and ~2000 Ω (T84). TER was measured using a Millicell ERS volt-ohm meter (Millipore, Bedford, MA, USA).

Human cervix transplant

Human cervix explants were obtained from National Disease Research Interchange (NDRI, PA, USA). Basically, 1/4 of cervix tissue was obtained from patients undergoing hysterectomy less than 24 hr. Cervix explants were then trimmed to get rid of most muscle tissue leaving the endocervix and ectocervix epithelium. Each piece was about 2.5 cm (L) X 0.6 cm (W) X 0.3 cm (H). These pieces were then incubated in CMRL-1066 (GIBCO, Grand Island, NY, USA) for 24 hr and switched to non-antibiotic media for experiment. The cut tissue was incubated with/without GC at MOI:10 for 24 hr with rinsing at time point 6 hr and 12 hr. The tissue was fixed and embedded in gelatin for cryosectioning. Sections were immunostained with desired antibodies for visualizing targeted proteins for their distribution.

Immunofluorescence analysis

Cells were pre-treated with or without NMII kinase inhibitors Y27632 and ML-7 (10 μM, Calbiochem, San Diego, CA, USA) or 2APB - inhibitor for IP3-induced calcium influx (Millipore, Temecula, CA, USA) for 1 hr, and incubated with GC in the presence or absence of the inhibitors for 6 h. Cell were washed and fixed with 4% paraformaldehyde, permeabilized, and stained with anti-ZO-1 (BD Bioscience, Bedford, MA, USA), anti-E-cadherin (BD Bioscience, Bedford, MA, USA), Phalloidin (Life Technology, Grand Island, NY, USA), anti-pMLC (Cell Signaling Technology, Danvers, MA, USA), and anti-GC [120] 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 0.37 μm 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 apical leakage of cellmask dye was determined by visual inspection of multiple images. The redistribution of actin and pMLC on apical surface was quantified by the fluorescence intensity ratio (FIR) of at GC adherent site to non-GC adherent site using fluorescence intensity profiles generated by ImageJ software.

3.3 Results

3.3.1 Polarized and non-polarized epithelial cell lines mimic the morphological and junctional properties of human ecto- and endo-cervical epithelium

The cervix is the gate between the vaginal region and the upper part of the female reproductive track. The cervical epithelium transitions from vaginal multi-layer squamous epithelium to endometrial single layer columnar epithelium, forming completely different ectocervial and endocervical epithelium. In order to mimic gonococcal infection in ectocervical epithelium (non-polarized epithelial cells) and endocervical epithelium (polarized epithelial cells), I used a human endometrial cell line HEC-1-B and a human colonic cell line T84 as epithelial cell models. Both of cell lines have been widely used in the gonococcal research field and are capable of polarizing in culture. To mimic ecto- and endo-cervical epithelial cell that are differentiated from the same origin [37, 139], I cultured HEC-1-B and T84 cells on

transwells for 2 days to generate non-polarized epithelial cells and for 8 days to generate polarized epithelial cells.

The hallmark between non-polarized and polarized epithelial cells is the presence of the apical junction. I first compared the cellular distribution of the apical junction proteins between ectocervical and endocervical epithelial cells. Human cervix tissue surgically removed via hysterectomy about 24 h earlier was cut into thin pieces along ecto- and endo-cervical epithelium. Tissue sample was fixed, infiltrated, and embedded in gelatin and frozen by liquid nitrogen. Cryosections (30 μm) were collected and stained for the tight junctional protein ZO-1, the adherens junctional protein E-cadherin, and F-actin. The tissue sections were analyzed by confocal fluorescence microscopy. In the ectocervical tissue, the epithelium consisted of a multilayer of epithelial cells, expanding up to 100 μm thickness (Fig. 14). The most outerlayer cells appeared to be shed from the tissue. In the endocervical tissue, the epithelium was a single layer of compacted epithelial cells with the thickness of 15-20 μm, and there was no shedding cells visible (Fig. 14).

In the ectocervical epithelial cells, the staining for the adherens junction protein E-cadherin was largely absent in the top 5-6 layer of ectocervical epithelium, and gradually increased in the cytoplasm of the inner layers of epithelial cells (Fig. 14A). In contrast, the E-cadherin staining in endocervical epithelium was distributed uniformly along the lateral membrane but not the apical membrane of the epithelial cells (Fig. 14A). The staining of tight junction protein ZO-1 was also absent in the ectocervical epithelium, while concentrating at the apical side of the basolateral membrane in the endocervical epithelium (Fig. 14B).

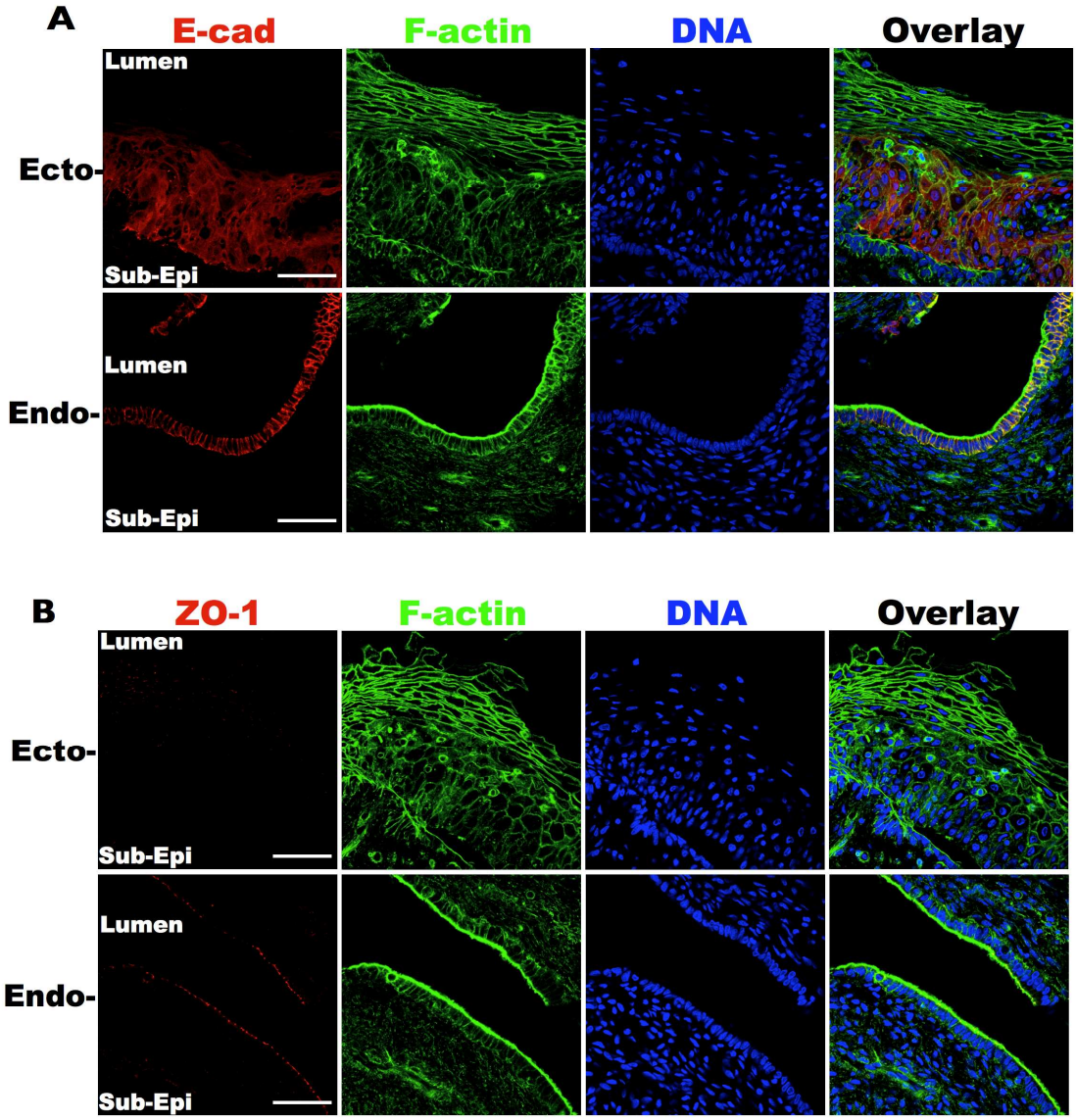

A
E-cad
F-actin
DNA
Overlay
Ecto-
Endo-
Lumen
Sub-Epi
B
ZO-1
F-actin
DNA
Overlay
Ecto-
Endo-
Lumen
Sub-Epi

Figure 14. Morphology and apical junction distribution in Ecto- and Endo-cervical epithelium

Cervix tissue explants were received, trimmed, and recovered in CMRL1066 media for 2 days. Tissue explants were then fixed, infiltrated and sectioned into 30 µm thickness. (**A)** Sectioned Ecto- and Endo-cervix were stained with adherens junction protein marker E-cadherin (red), F-actin (green) and nucleus (blue). (**B)** Sectioned Ecto- and Endo-cervix were stained with tight junction protein maker ZO-1 (red), F-actin (green) and nucleus (blue). Bar = 50 µm

In order to mimic GC infection at the ecto- and endo-cervix that contain polarized and non-polarized epithelia from the same cell origin, I used the human endometrial epithelial HEC-1-B cell line and the human colonic epithelial T84 cell line as the models because they are the only biologically relevant cell lines that can be polarized in culture and they are widely used for gonococcal infection research. I cultured T84 or HEC-1-B cells on transwell inserts for 2 days to mimic non-polarized epithelial cells and for 10 days to mimic polarized epithelial cell properties. To characterize their polarity, I examined the distribution of the apical junctional proteins, using 3-dimensional fluorescence microscopy. The 2-day cultured HEC-1-B cells were flat compared to the 10 day cultured cells (Fig. 15A). E-cadherin staining showed low-level and sporadic distribution in the cytoplasm in the 2-day cells but more concentrated at the lateral membrane in the 10-day cells. ZO-1 staining was absent in the cytoplasm and some staining at the cell-cell contact in the 2-day cells where it is concentrated near the border between the apical and basolateral membrane (Fig, 15A).

Similarly, the 2-day T84 cells were flat whereas the 10-day cells were tall (Fig. 15B). E-cadherin staining showed uneven distribution with most localized at the lateral side of cells in the 2-day cells, but was concentrated at lateral membrane in the 10-day cells. ZO-1 staining was absent in the 2-day cells and appeared in the 10-day cells at the cell membrane of the apical/lateral boarder, similar to epithelial cells in the endocervix (Fig. 15B).

Taken together, the ecto- and endo-cervical epithelial epithelial cells exhibit typical characteristics of non-polarized and polarized epithelial cells. HEC-1-B and T84 cell

line growing on transwells for 2 and 10 days exhibit the morphological and cell-cell junctional characteristics of non-polarized and non-polarized epithelial cells, similar to ecto- and endo-cervical epithelial cells.

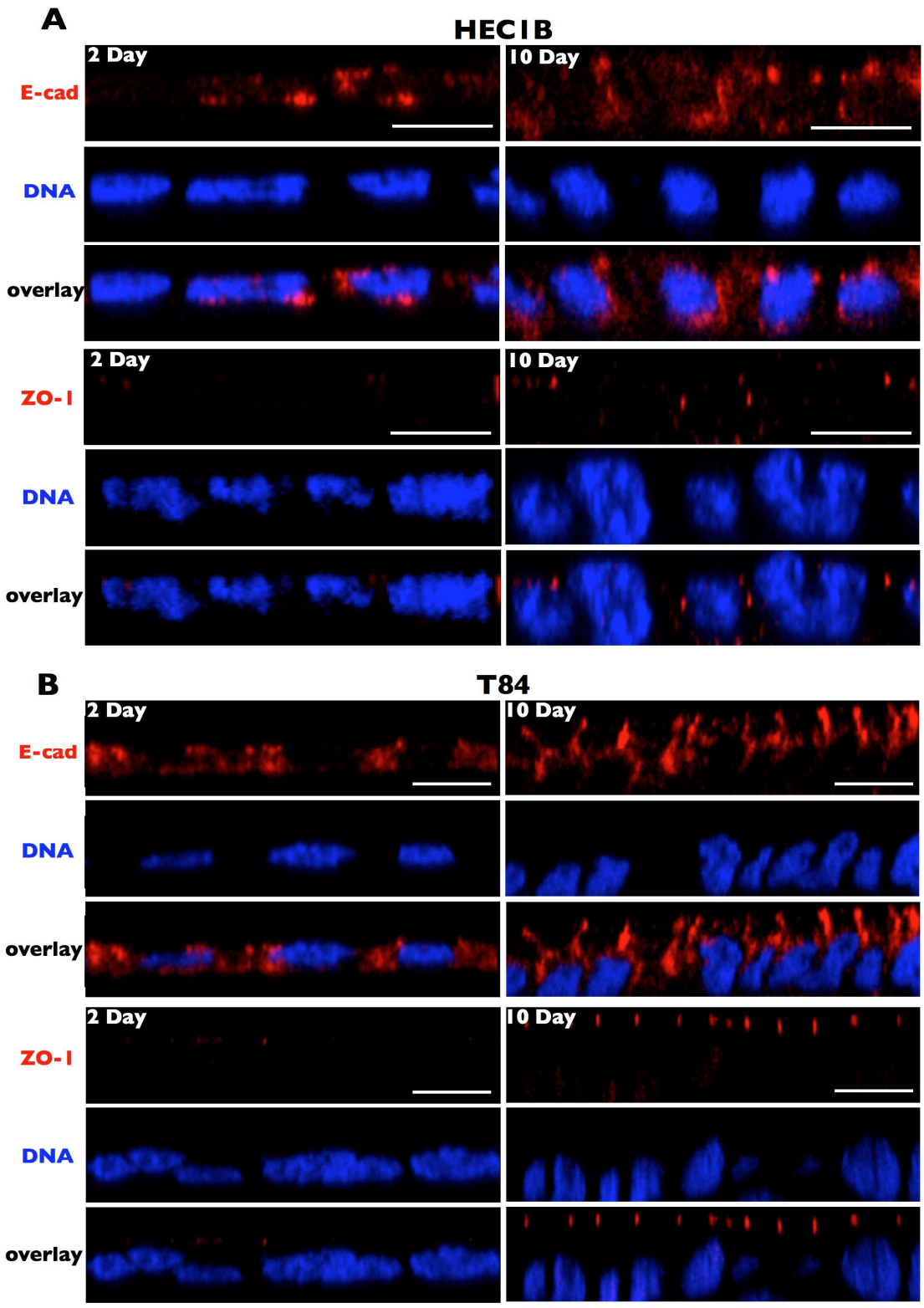

A
HEC1B
2 Day
10 Day
E-cad
DNA
overlay
2 Day
10 Day
ZO-1
DNA
overlay
B
T84
2 Day
10 Day
E-cad
DNA
overlay
2 Day
10 Day
ZO-1
DNA
overlay

Figure 15. Apical junction distribution in non-polarized and polarized HEC-1-B and T84 cell line

HEC-1-B and T84 cells were seeded into transwell inserts for 2 days and 10 days separately to represent low polarity and high polarity. (**A**) 2 day and 10 day HEC-1-B cells were stained with adherens junction protein maker E-cadherin (red) or tight junction protein maker ZO-1 (red) and nucleus (blue). (**B**) 2 day and 10 day T84 cells were stained with t adherens junction protein maker E-cadherin (red) or tight junction protein maker ZO-1 (red) and nucleus (blue). Bar = 10 μm

3.3.2 Functional differences in the cell-cell junction between non-polarized and polarized HEC-1-B and T84 epithelial cells

Epithelial cells provide the physical barrier against pathogen invasion. The apical junction is critical for this barrier function by sealing the paracellular space of columnar epithelial monolayers. The apical junction has two functions - gate and fence function, which regulate the permeability of paracellular space and the diffusion of lipids and membrane proteins between the apical and basolateral surfaces, respectively. In order to examine the barrier function of HEC-1-B and T84 cells, I used transepithelial electric resistance (TEER) to monitor the permeability of the paracellular space, and live cell imaging of membrane dyes to visualize lateral diffusion between the basolateral and apical membrane.

The transepithelial electric resistance between the apical and basal chambers was measured by a Millicell® ERS Voltohmmeter. The TEER of the 2 day HEC-1-B cells was at 150 Ω/cm2 and reached 370 Ω/cm2 at 10 days. The TEER of T84 cells at 2 day reached ~400 Ω/cm2 and 3700 Ω/cm2 at 10 days (Fig. 16A). For analyzing fence function, epithelial cells were incubated with the CellMask lipid dye in the basolateral chamber. The polarized distribution of the basolaterally stained CellMask dye at the basolateral surface indicates the integrity of the fence function. In the 2 day HEC-1-B cells, the basolaterally labeled CellMask appeared at both basolateral and apical membrane, but displayed at the basolateral membrane only in the 10-day cells (Fig. 16B). I quantified the results by visually counting the number of cells with the apical membrane positive for CellMask staining. More than 90% of the 2-day HEC-1-B cells showed the basolateral to apical diffusion while only 25% of the 10 day HEC-1-

B cells showed so. Similarly, 75% of the 2 day T84 cells had the CellMask dye in the apical surface, while only 10% of the 10 day T84 cells did so (Fig. 16C). In order to quantify the level of lateral leakage, I measured the fluorescence intensity ratio (FIR) of the CellMask dye staining at the apical to that at lateral membrane. The 2-day HEC-1-B cells had a ratio of 0.9, indicating a similar level of the CellMask staining at the apical and lateral membrane wheres the 10-day HEC-1-B cells had a FIR of 0.3, showing that about 25% of the lateral CellMask staining diffusing into the apical membrane. The FIR of the 2-day T84 cells was 1.1 and decreased to 0.03 in the 10-day cells (Fig. 16D), indicating a much stronger fence function of polarized T84 cells than that in polarized HEC-1-B cells These results suggest HEC-1-B and T84 epithelial cell line, in different day growth, can reach different levels of polarity and gate and fence functions. Although HEC-1-B and T84 cells reach different level of polarity, upon reaching the maximal TEER these two cell lines have all the characteristics of polarized columnar epithelial cells with distinct junction protein distribution and functionality. The cells cultured on transwells for 2 days represent all the characteristics of non-polarized epithelial cells. This culture model system allows us to use epithelial cell lines to mimic different polarity levels of cervical epithelium in lower female reproductive tract.

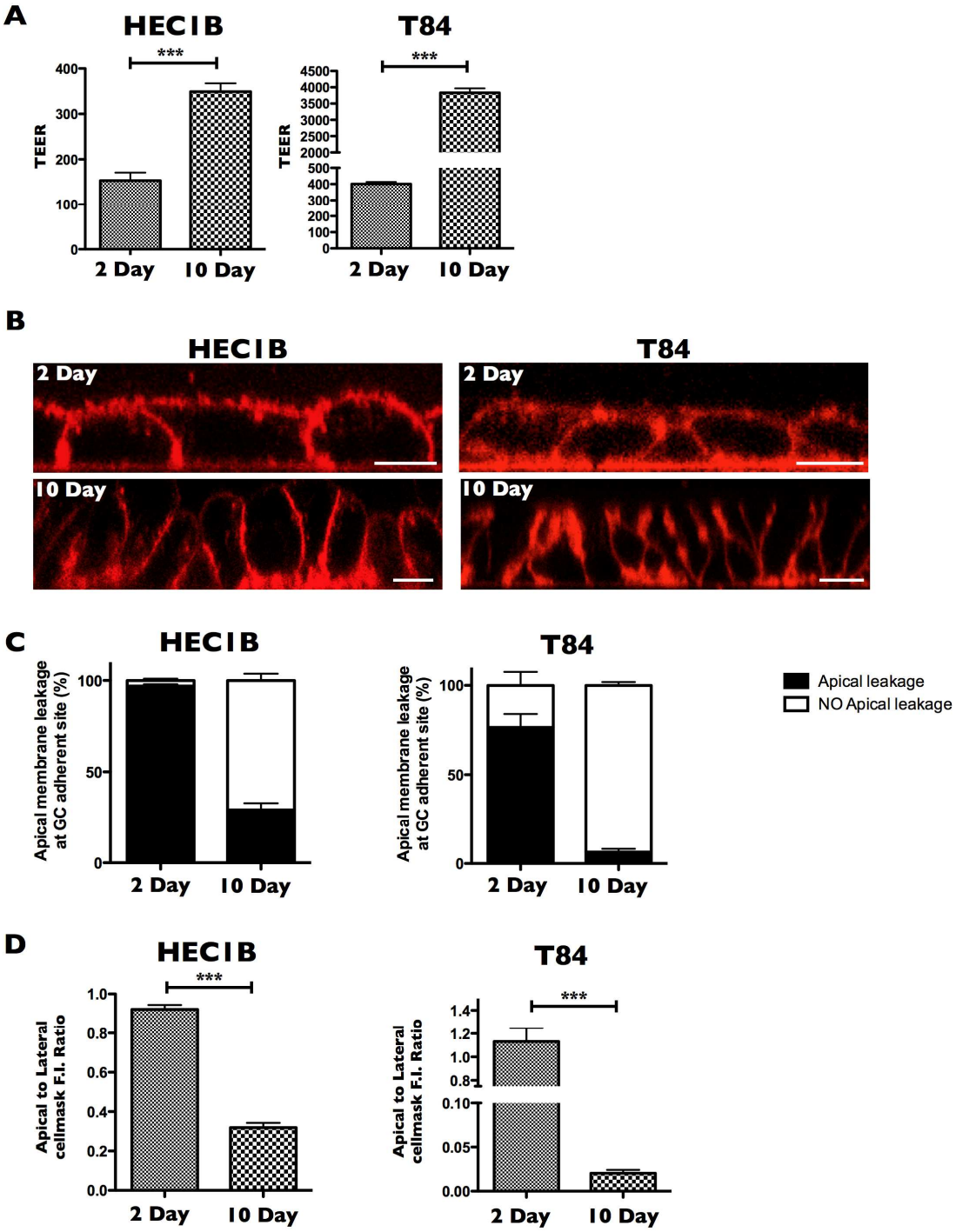

A
HEC1B
T84
***
***
TEER
TEER
2 Day
10 Day
2 Day
10 Day
B
HEC1B
T84
2 Day
2 Day
10 Day
10 Day
C
HEC1B
T84
Apical membrane leakage at GC adherent site (%)
Apical membrane leakage at GC adherent site (%)
2 Day
10 Day
2 Day
10 Day
Apical leakage
NO Apical leakage
D
HEC1B
T84
***
***
Apical to Lateral cellmask F.I. Ratio
Apical to Lateral cellmask F.I. Ratio
2 Day
10 Day
2 Day
10 Day

Figure 16. Junction functionality in non-polarized and polarized HEC-1-B and T84 cell line

HEC-1-B and T84 cells were seeded onto transwell inserts invertedly and grew for 2 days and 10 days separately to represent low polarity and high polarity. (**A)** TEER was measured in HEC-1-B (left) and T84 cells (right). (**B)** CellMask (red) lipid dye was applied to basolateral membrane and cells were imaged in XZ view for lipid diffusion over apical junction on HEC-1-B (left) and T84 cells (right). Bar = 10 μm (**C)** Quantification was performed by the analysis of cells with/without CellMask (red) lipid dye diffusion to the apical part. HEC-1-B (left) and T84 cells (right). (**D)** Analysis of CellMask (red) lipid dye diffusion from basolateral to apical membrane by quantifying apical to lateral fluorescence intensity ratio. Shown are the average FIR of CellMask staining in >40 individual cells of three independent experiments. ***p ≤0.001; **p ≤ 0.01; *p≤0.05.

3.3.3 Gonococcal interaction induce different actin remodeling in polarized and non-polarized epithelial cells

The actin cytoskeleton is known to be one of the host factors that are required for gonococcal infection. It has been reported that actin is recruited during GC infection in epithelial cells [63, 66, 140]. The organization of actin is drastically different between non-polarized and polarized epithelial cells in the ecto- and endo-cervical epithelium or in cell line culture. There hasn't been any reported study comparing GC infection between non-polarized and polarized epithelial cells. Here I examined the effect of gonococcal infection on the actin distribution in non-polarized and polarized epithelial cells. T84 cells cultured on transwell inserts for 2 or 10 days were incubated in the presence or absence of GC. Cells were then fixed and stained for GC and F-actin.

The 3D confocal fluorescence microscopic analysis showed that actin was distributed evenly at the periphery of the 2-day cultured T84 cells. There was no difference in F-actin levels at the cell membrane contacting and away from the transwell filter. In contrast, F-actin was highly concentrated at the apical membrane in the 10-day T84 cells. These data indicate that the actin distribution in the 2-day and 10-day T84 cells was similar to that in ecto- and endo-cervical epithelium (Fig 17A & 17B). In the presence of GC, F-actin was recruited to GC adherent sites with ~2.3 fold higher mean fluorescence intensity of F-actin at GC adherent sites than at non-GC site (Fig. 17A). In contrast, the F-actin staining level at GC adherent sites of the apical surface in the 10-day T84 cells was ~1 fold lower, compared to non-GC sites (Fig 17B-C). In

addition, the spatial relationship between adhered GC with the 2 day and 10 day T84 cells is different. Adherent GC induced membrane ruffled in the 2-day T84 cells surrounding the spherical GC microcolony. However, in 10-day T84 cells the adherent GC sunk into the apical membrane with F-actin at the side, but not at bottom, of GC and form loose clusters (Fig 17A-B). The results indicate that GC induce different actin remodeling and form different interactions with non-polarized and polarized epithelial cells.

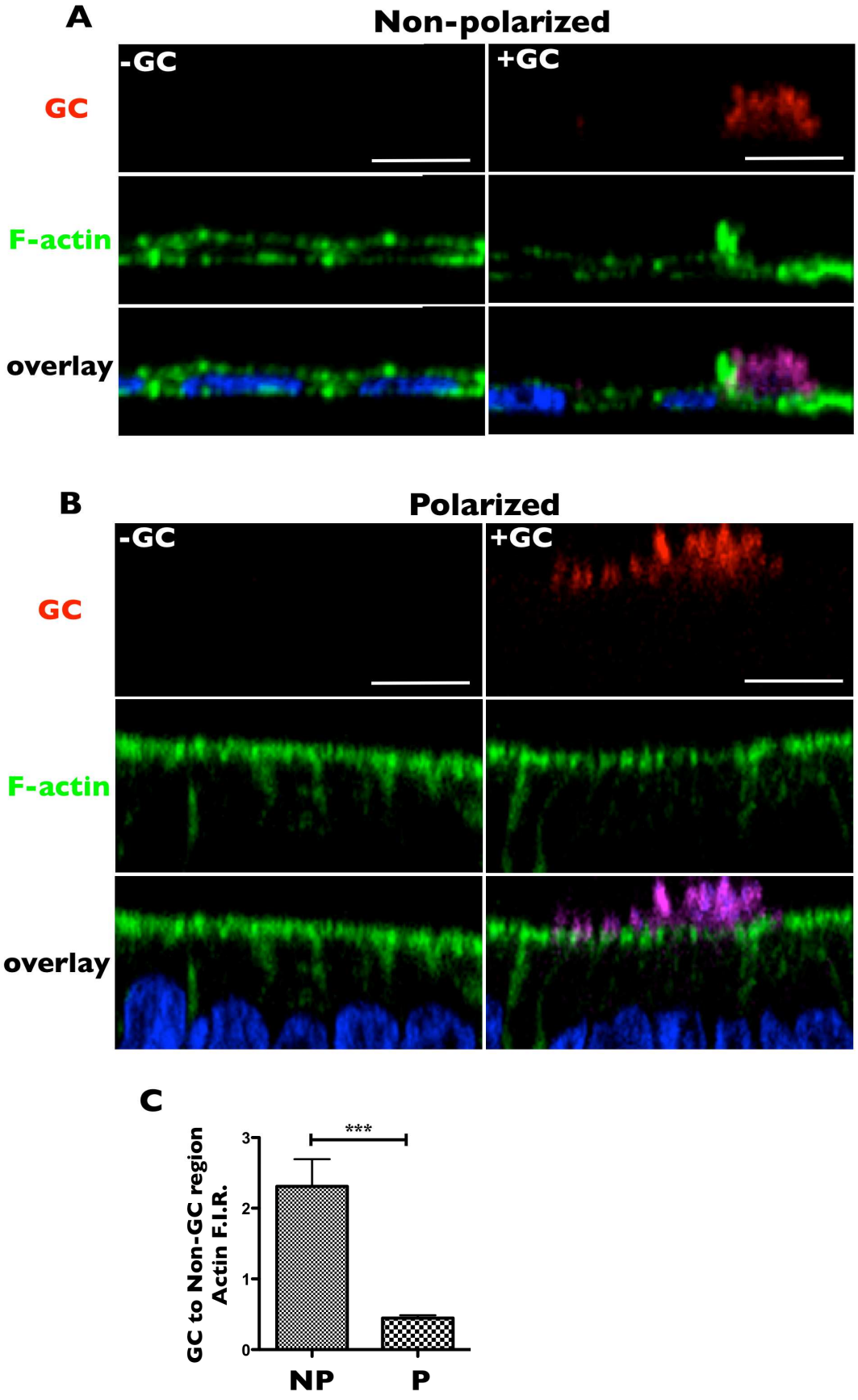

A
Non-polarized
-GC
+GC
GC
F-actin
overlay
B
Polarized
-GC
+GC
GC
F-actin
overlay
C
GC to Non-GC region Actin F.I.R.
***
3
2
1
0
NP
P

Figure 17. Actin distribution between non-polarized and polarized T84 cells in the presence and absence of GC

T84 cells were seeded onto transwell inserts for 2 days and 10 days separately to represent non-polarized and polarized epithelium. (**A**) Non-polarized T84 cells were incubated in the media with or without GC. Cells were fixed and stained with antibody against GC (red), F-actin (green) and nucleus (blue). (**B**) Polarized T84 cells were incubated in the media with or without GC. Cells were fixed and stained with antibody against GC (red), F-actin (green) and nucleus (blue). Bar = 10 μm (**C**) Quantification of F-actin mean fluorescence intensity ratio (GC site to non GC site) on T84 cells with GC inoculation. NP: Non-polarized T84, P: Polarized T84. Shown are the average FIR of F-actin staining in >40 individual cells of three independent experiments. ***p ≤0.001; **p ≤ 0.01; *p≤0.05.

3.3.4 NMII contributes to actin distribution differences in polarized and non-polarized epithelial cells

Non-muscle myosin II (NMII), an actin motor protein that contracts actin when activated, has also been shown to disassemble actin in keratinocytes [69]. In the previous chapter, I have shown that gonococcal apical inoculation induces NMII redistribution in polarized cells and this activation contributes to apical junction disruption and gonococcal transmigration. Here I address whether GC interaction with non-polarized also induced similar NMII redistribution and whether NMII redistribution contributes to the differential actin remodeling in non-polarized and polarized epithelial cells. I compared the distribution of activated NMII in non-polarized and polarized epithelial cells with/without gonococcal inoculation and the effect of inhibition of NMII activation influences the actin remodeling in epithelial cells. Using 3D confocal fluorescence microscopy to examine the distribution of phosphorylated NMII light chain (pMLC).

In the absence of GC, pMLC staining was distributed evenly at both the cell periphery and the cell-cell contact in the 2-day T84 cells, whereas pMLC was primarily concentrated at the apical junction in the 10-day T84 cells (Fig 18A & 18B). Incubation with GC for 6 h did not significantly change the distribution of pMLC, and there were similar levels of pMLC staining at GC adherent site compared to those in non-GC site and in the 2-day cells without GC inoculation (Fig. 18A). After apical incubation with GC for 6 h, the 10-day cells with GC showed the recruitment of pMLC at GC adherent site (Fig. 18B). To quantify the redistribution of pMLC, I measured the fluorescence intensity ratio (FIR) of pMLC at GC adherent

sites to non-GC sites. The FIR in the 10-day epithelial cells 1.8 was much higher than that of the 2-day cells 0.8 (Fig. 18C), quantitatively confirming the recruitment of active NMII to GC in polarized, but not non-polarized, epithelial cells.

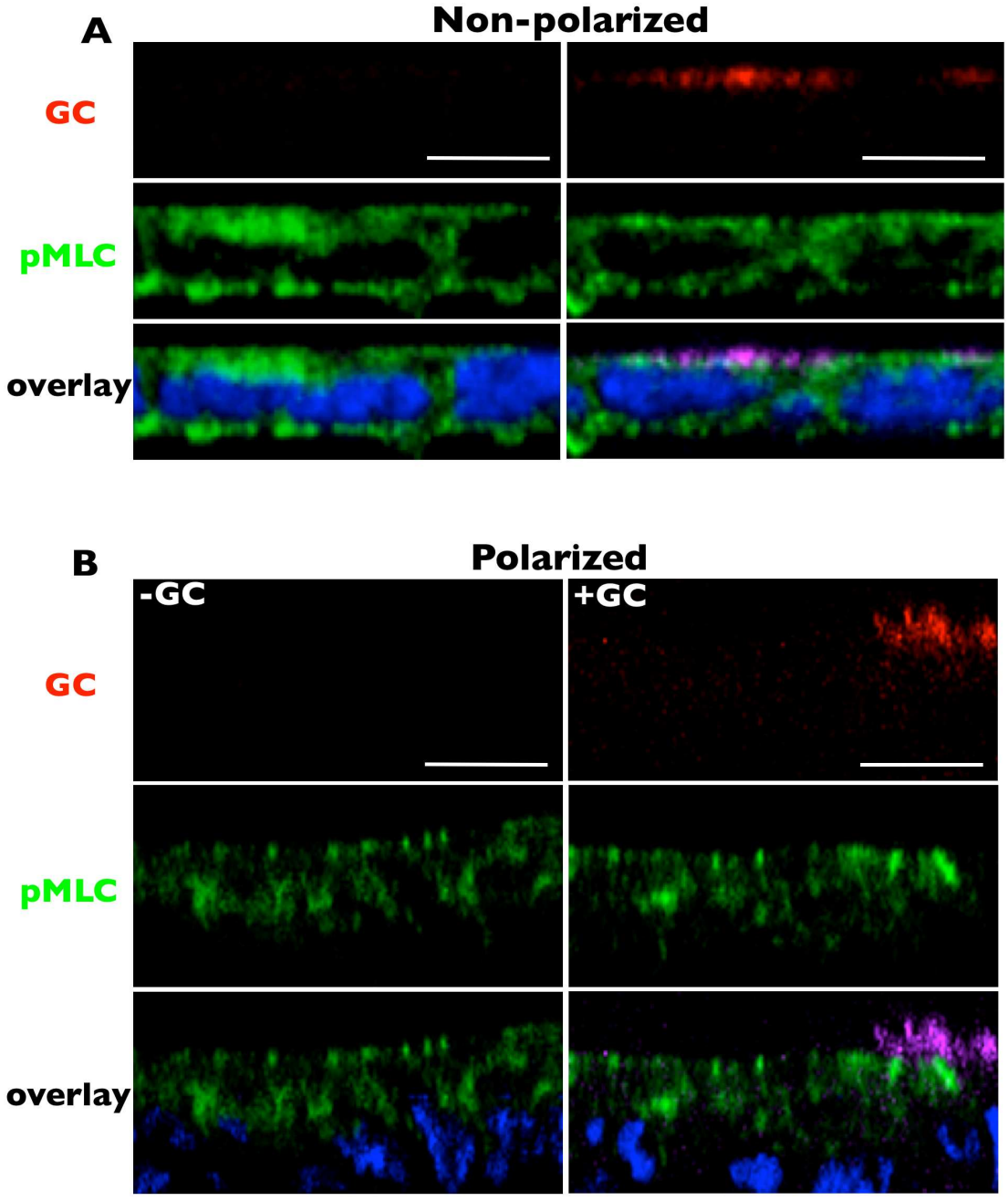

A
Non-polarized
GC
pMLC
overlay
B
Polarized
-GC
+GC
GC
pMLC
overlay
C
***
GC to Non-GC region pMLC F.I.R.
2.5
2.0
1.5
1.0
0.5
0.0
NP
P

Figure 18. pMLC distribution between non-polarized and polarized T84 cells in the presence and absence of GC

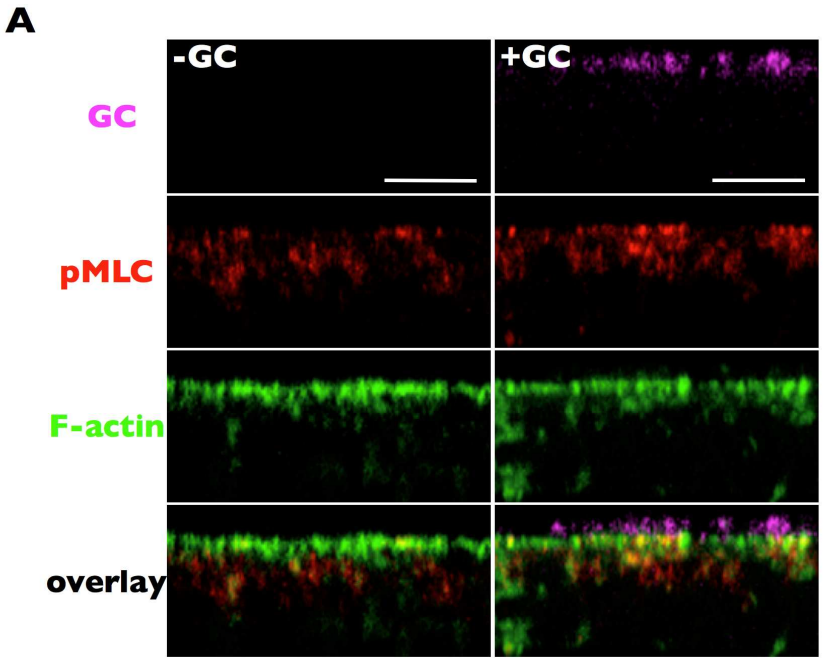
T84 cells were seeded onto transwell inserts for 2 days and 10 days separately to represent non-polarized and polarized epithelium. (**A)** Non-polarized T84 cells were incubated in the media with or without GC. Cells were fixed and stained with antibody against GC (red), pMLC (green) and nucleus (blue). (**B)** Polarized T84 cells were incubated in the media with or without GC. Cells were fixed and stained with antibody against GC (red), pMLC (green) and nucleus (blue). Bar = 10 μm (**C)** Quantification of pMLC mean fluorescence intensity ratio (GC site to non GC site) on T84 cells with GC inoculation. NP: Non-polarized T84, P: Polarized T84. Shown are the average FIR of pMLC staining in >40 individual cells of three independent experiments. ***p ≤0.001; **p ≤ 0.01; *p≤0.05.

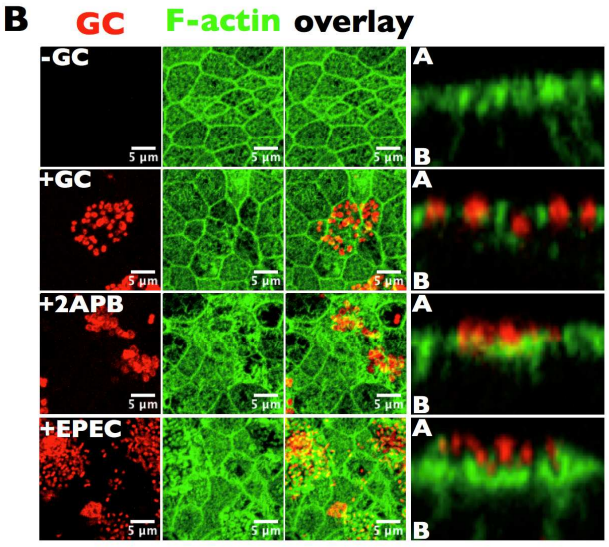
In order to investigate if the NMII recruitment is involved in the decrease in the F-actin level under GC adherent sites in polarized epithelial cells, I first analyzed the spatial relationship between pMLC and F-actin in the 10-day T84 cells with or without GC inoculation. In the absence of GC, pMLC was partially colocalized with F-actin at the apical surface but was not colocalized with F-actin in the cytoplasm. In the cells inoculated with GC, the fluorescence intensity of pMLC was increased while the fluorescence intensity of F-actin decreased under GC adherent sites, leading to reduced colocalization between pMLC and F-actin (Fig. 19A). These data suggest a correlation between the recruitment of active NMII and the reduction of F-actin at GC adherent sites. The colocalization between pMLC and F-actin will need to be quantified by Pearson's correlation coefficient in the future.

To investigate the relationship between active NMII and F-actin, I inhibited NMII activation using ROCK inhibitors (Y27632), MLCK inhibitor (ML-7), and the Ca^{2+} inhibitor 2APB to examine their effects on GC-induced actin remodeling in polarized epithelial cells, using 3D immunofluorescence microscopy. I found that treatment of Y27632, ML-7 (data not shown), or 2APB inhibited the F-actin loss and restored F-actin levels under GC adherent sites (Fig. 19B). The inhibition of NMII activation significantly increased the F-actin FIR of GC adherent to non-GC site (Fig. 19C).

Taken together, these results indicate that GC can induce the recruitment of active NMII to adherent sites in polarized but not in non-polarized epithelial cells, and active NMII at GC adherent sites causes a reduction in the F-actin level, probably via

NMII contraction-induced actin depolymerization. It showed NMII is involved in more than one phenomenon induced by GC in polarized epithelial cells. Different from junction regulation by MLCK in polarized epithelial cells, it suggested that both ROCK and MLCK are involved in NMII activation for actin disassembly by GC. The role of NMII in non-polarized epithelial cells still needs to be determined.


A
-GC
+GC
GC
pMLC
F-actin
overlay


B
GC
F-actin
overlay
-GC
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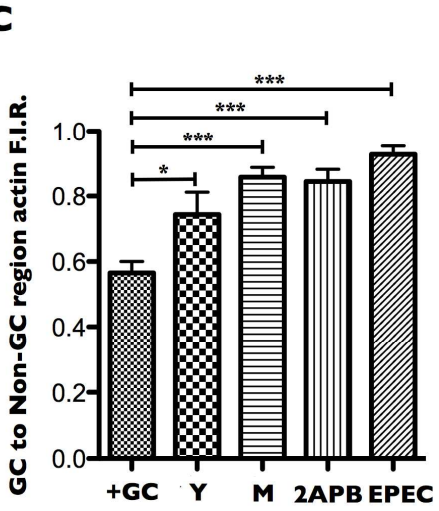
+2APB
+EPEC
5 µm
A
B


C
GC to Non-GC region actin F.I.R.
1.0
0.8
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0.4
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***
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*
+GC
Y
M
2APB
EPEC

Figure 19. NMII contributes to actin disassembly in polarized T84 cells in the presence of GC

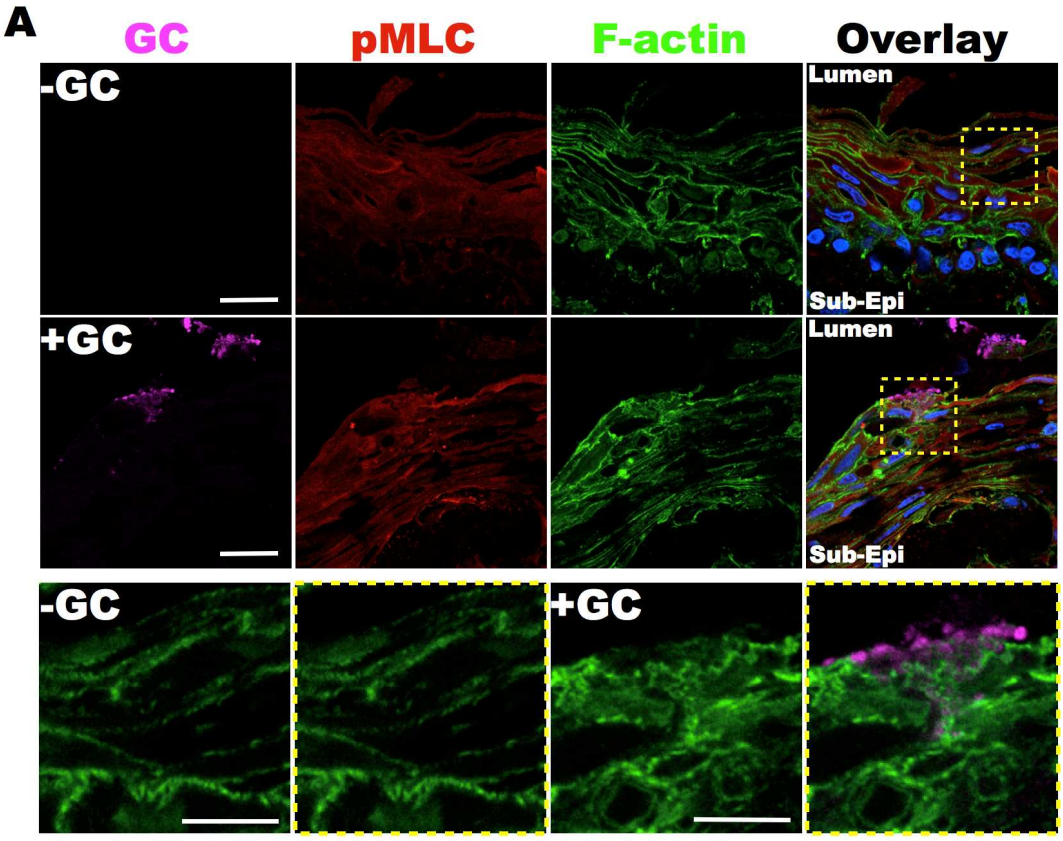
T84 cells were seeded onto transwell inserts for 10 days separately to form polarized epithelial layer. **(A)** Polarized T84 were incubated in the media with or without GC. Cells were fixed and stained with antibody against GC (magenta), pMLC (red), and F-actin (green). Bar = 10 μm **(B)** Polarized T84 cells were incubated in the media with GC in the absence or presence of NMII inhibitors. Cells were fixed and stained with antibody against GC (red) and F-actin (green). Bar = 5 μm **(C)** Quantification of GC to Non-GC region F-actin mean fluorescence intensity ratio with GC inoculation in the presence or absence of NMII inhibitors. Shown are the average FIR of F-actin staining in >40 individual cells of three independent experiments.

***p ≤0.001; **p ≤ 0.01; *p≤0.05. Y: Y27632 M: ML-7

3.3.5 Gonococcal infection in human cervical transplant

To confirm GC-induced differential actin remodeling in epithelial cells, I examined the impact of GC inoculation on the distribution of F-actin and pMLC in epithelial cells of ecto- and endo-cervical tissue explants described above. The tissue pieces [2.5 cm (L) x 0.6 cm (W) x 0.3 cm (H)] containing ecto- and endo-cervical epithelium were incubated with or without GC at a MOI of 10 for 24 h with washes at 6 hr and 12 hr to remove non-adherent bacteria. The tissue was then prepared for cryosection. Sections were immunostained for GC, F-actin and pMLC, and analyzed using confocal fluorescence microscopy. In the ectocervical epithelium, F-actin was distributed evenly at the periphery of the multi-layered squamous epithelial cells while pMLC was evenly distributed in the cytoplasm. In the presence of GC, F-actin was recruited to GC adherent site and forms phagocytic cup-like structures. The distribution of pMLCdid not appear to be changed by GC adherence, but the overall pMLC level appeared to be higher than that in the ectocervical epithelial cells without GC inoculation (Fig. 20A). In the endocervical epithelium, F-actin was primarily concentrated at the apical membrane and apical junction, while pMLC was concentrated at the basal surface. Similar to GC-inoculated polarized cell lines in culture, the F-actin staining level was decreased and the pMLC staining level was increased exclusively at GC adherent sites of the endocervical epithelial cells (Fig. 20B). The FIR of GC adherent sites to non-adherent sites confirmed a significant decrease in the F-actin level and a significant increase in the pMLC level under GC adherent sites (data not shown). These results indicate GC induce actin remodeling in

different manners in the ecto- and endo-cervical epithelial cells, similar to what I observed in epithelial cell lines cultured in non-polarized and polarized conditions. The differential actin remodeling suggests that GC generate different infection strategies when infecting ecto- and endocervical epithelium.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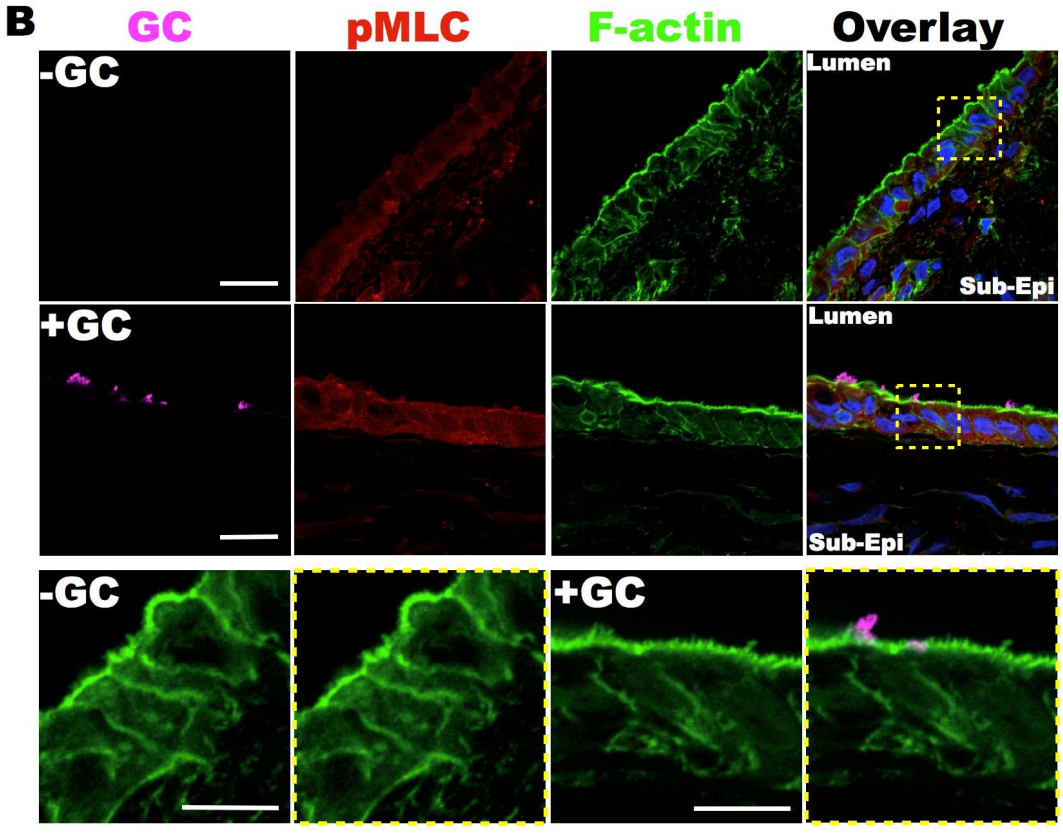
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Figure 20. Actin and NMII distribution in Ecto- and Endo- cervical epithelium in the presence and absence of GC

Cervix tissue explant with ecto- and endo- cervix was processed for GC infection at MOI:10. Cervix tissue explants were incubated in the media with or without GC. Cells were fixed, sectioned and stained with antibody against GC (magenta), pMLC (red), and F-actin (green). (**A)** Ectocervix (**B)** Endocervix. (Top panel: Bar = 20 μm, Bottom panel: Bar = 10 μm)

3.4 Discussion

In this study, I have established and characterized human cervical tissue explants. Using these tissue explants, I compared the endocervical and ectocervical epithelial cells. My results show the differential actin organization and junction complex formation between non-polarized squamous ectocervical and polarized columnar endocervical epithelial cells. I found that non-polarized and polarized human endometrial HEC-1-B cells and colonic T84 cells cultured on transwell filter mimic endo and ecto-cervix epithelial cells in culture. Using both model systems, my studies demonstrate that gonococcal apical inoculation induces differential actin remodeling: the accumulation of F-actin at GC adherent sites in non-polarized epithelial cells in culture and in human ectocervical tissue plants, and NMII-dependent reduction of F-actin beneath GC adherent sites in polarized epithelial cells in culture and in human endocervical tissue explants. The differential actin remodeling is associated with the extensive interaction of GC microcolonies with non-polarized epithelial cells and GC-induced apical junction disruption in polarized epithelial cells.

It is well known that HEC-1-B and T84 cells cultured in the same conditions exhibit different levels of cell polarity, which may be due to the original tissues from which are derived and the levels of growth factor receptors, such as ErbB family receptors, that control their proliferation rate. Despite their differences in polarity, the 10-day culture of HEC-1-B and T84 cells on transwells exhibited the polarized distribution of apical junction proteins similar to the endocervical epithelial cells in the human tissue explants, while the 2-day cultured epithelial cell lines on transwells show no or non-

polarized distribution of junctional proteins, similar to ectocervical epithelial cells in the tissue explants. By analyzing the barrier functional of the apical junction (gate and fence), I provide a quantitative evaluation of the polarity of the two cell lines under non-polarized and polarized culture conditions.

This study shows that GC-induced differential actin remodeling in non-polarized and polarized epithelial cells also occurs in the human ectocervical and endocerical epithelial cells, suggesting that the same remodeling may happen *in vivo*. The cervical epithelial cells, coming from the same origin, develop into two different types: the ecto- and endo-cervical epithelium [37, 139]. Based on our and others data, one of the most significant difference between ecto- and endo-cervical epithelial cells is the polarity. The differences in polarity affects cell morphology, functionality, intracellular signaling, and the response to pathogens differently. I have shown that HEC-1-B and T84 cells cultured for 2 and 10 days display no or strong polarity respectively, thereby representing the ecto- and endo-cervical epithelial cells and being good models for GC infections. However, due to their cancerous nature, there are various potential differences between HEC-1-B and T84 cells and human cervical epithelial. For example, the signaling that drives continuous proliferation in cell lines may not be constitutively activated in the cervical epithelial cells [141]. The endocervical epithelial cells but not the cell lines produce and secrete a large amount of mucus. The cervical epithelial cells are also sensitive to sex hormones. The fluctuation of estrogen and progesterone affects the signaling and survival of cervical epithelial cells [142]. The non-polarized and polarized HEC-1-B and T84 cells

provide models for examining how epithelial cell polarity influences GC pathogenesis. However, we should be mindful about the differences between epithelial cell lines and epithelial cells in human cervix.

The most significant finding of this study is the differential reorganization of actin cytoskeleton and the actin contractile motor NMII induced by GC in non-polarized and polarized T84 model under GC infection. Actin is recruited in non-polarized epithelial cells, but reduced in polarized epithelial cells at GC adherent sites. The activated NMII is recruited to GC adherent site in polarized, but not in non-polarized epithelial cells. By inhibiting NMII activation, I demonstrate that the reduction of F-actin under GC adherent sites in polarized epithelial cells is dependent on the recruitment of active NMII, suggesting that NMII-mediated actin contraction leads to actin disassembly. While little is known concerning how GC differentially regulate actin remodeling in polarized and non-polarized epithelial cells, it is likely the results of differential organization of actin and differential signaling regulation of actin remodeling in polarized epithelial cells.

The actin cytoskeleton is one of the critical host factors that GC take advantage for infection. Previous studies have shown that GC can recruit actin on host epithelial cells for invasion. However, most of these results were generated using non-polarized epithelial cells. Actin organization is different in ecto- and endocervical epithelial cells. In ectocervical epithelial cells, actin distributes on the cell periphery without significant polarization, which supports cell morphology. In endocervical epithelial

cells, F-actin is highly concentrated at the apical membrane and perijunctional location. This high concentration of F-actin in the apical surface supports microvilli (such as the brush boarder) and the apical junction requires a perijunctional actomyosin belt that associates with the junction complex via adaptor protein catenins and ZO-1. The recruitment of F-actin to GC in non-polarized epithelial cells requires the activation of actin polymerization at GC adherent sites, while the disassembly of the apical and perijunctional actin belt required the detachment and depolymerization of actin. These two modes of actin remodeling are very likely to be induced by different upstream signals and require different actin regulators. Consequently, GC form different interactions with non-polarized and polarized epithelial cells. The recruitment of F-actin for microvilli elongation and eventual bacterial internalization is a common mechanism for bacterial invasion [65]. However, bacterial-induced actin disassembly in the apical surface of polarized epithelial cells has not been previously reported. A consequence of the apical actin disassembly is the loss or shortening of microvilli [143].

How GC induces redistribution of active NMII in polarized but non-polarized epithelial cells is not fully understood. One potential reason is the cell polarity. Signaling, actin, and their regulators in the cytoplasm and cell surface are distributed in different fashion in non-polarized and polarized epithelial cells, thereby potentially being activated at different levels and different locations and in different ways by GC. The potential role of NMII in depolymerization of F-actin in GC-infected polarized epithelial cells is supported by previous studies that demonstrated a role for NMII in

actin depolyermization in migrating keratinocytes [69]. *Ivanov et al*. showed a role for NMII in the disassembly of the perijunctional actomyosin belt via actin contraction. These studies together suggest two roles for NMII in GC interaction with polarized epithelial cells: disassembling the actin cytoskeleton under GC adherent sites and disrupting the apical junction, which facilitate GC infection.

My finding that inhibiting NMII activation by the ROCK inhibitor Y27632, the MLCK inhibitor ML-7, or the Ca^{2+} inhibitor 2APB inhibits GC induced actin disassembly suggests that GC-induced NMII activation is involved and both ROCK and MLCK as well as Ca^{2+} signaling. In contrast, GC-induced junction disassembly appears to only depend on MLCK. It suggests that NMII mediating actin depolymerization at GC adherent sites and the disassembly of the perijunctional actomyosin belt is not activated in the exactly same way. The significance of the apical actin disassembly to GC infection remains unclear. One hypothesis is that actin depolymerization would reduce the rigidity of the apical membrane and enable GC to adhere the apical surface of polarized epithelial cells more efficiently. Another possibility is to regulate mucus secretion by polarized endocervical epithelial cells. The endocervical epithelial cells secret mucus constantly by exocytosing mucus-containing vesicles from the apical surface, which provides another layer of protection against GC infection. Both apical actin disassembly and junction disruption may inhibit mucus secretion, facilitating GC adherence.

In summary, my results demonstrate that GC interaction induced different remodeling of the actin cytoskeleton in non-polarized and polarized epithelial cells in culture and in the ecto- and endo-cervical tissue explants. The differential actin remodeling leads to different morphological and functional changes in non-polarized and polarized epithelial cells, which suggest that GC utilize different mechanisms to establish infection at the ectocervix and endocervix. For the future research, I will focus on GC-induced signaling pathways that lead to the differential actin remodeling in non-polarized and polarized epithelial cells.

Chapter 4: Conclusions

4.1 Summary

Neisseria gonorrhoeae is an obligate pathogen of humans and causes significant health issues in the U.S. and worldwide. Gonorrhea is the second most commonly reported sexually transmitted infection in the U.S. Infected women are generally asymptomatic or subclinical, thus allowing infections to become chronic. Chronic infection increases the risk of serious complications, including PID and DGI. PID can results in scarring of the reproductive organs, ectopic pregnancy, and infertility can occur.

GC infect epithelial cells of the urogenital tract of both men and women. In the female reproductive track, the properties of epithelial cells change at different anatomic locations, including non-polarized and polarized. The cervical epithelium is suggested to be the initial infection site, and it consists of both non-polarized and polarized epithelial cells lining the surface of ectocervix to endocervix respectively. As ecto- and endo-cervix epithelial cells have different properties and change their properties with the hormonal environment, GC need to adapt to this ever changing host cells to establish infection.

The goal of this study was to gain a better understanding of the host signaling mechanisms that are essential for gonococcal interaction with and establish infection

at the genital epithelial cells of the female reproductive tract. The experiments were designed with an approach in order to investigate mechanisms by which GC manipulate the barrier function of the genital epithelial cells. My research found that GC induces NMII activation and recruitment to the apical surface and junction in polarized epithelial cells. GC activates NMII by inducing EGFR-dependent Ca^{2+} flux. This GC-induced Ca^{2+} flux is required for gonococcal-induced junction disruption and gonococcal transmigration. Ca^{2+} flux leads to MLCK activation, which in turn activates NMII. NMII-mediated contraction of perijunctional actin belt then induces junction disruption. Gonococcal WT MS11 and Δopa mutant both are able to induce NMII activation and in an MLCK-dependent manner. However, Δopa GC induces NMII activation to a greater magnitude than wt GC. This suggests that Opa plays a negative role in NMII activation, consequently interfering with GC-induced junction disruption and gonococcal transmigration across polarized epithelial cells.

To understand how GC establishes infection in the female reproductive epithelium, I established human cervical tissue explants as well as non-polarized and polarized epithelial cells in culture to mimic the non-polarized squamous ectocervical epithelial cells and the polarized columnar endocervical epithelial cells, respectively. My data showed that GC interaction induces differential remodeling of the actin cytoskeleton in non-polarized and polarized epithelial cells in human ecto- and endo-cervical tissue explants and in culture. The actin cytoskeleton is recruited under the GC adherent site in non-polarized epithelial cells and disassembled in polarized epithelial cells. The differential actin remodeling is dependent on the redistribution of activated NMII

through MLCK and ROCK and leads to different changes in the morphology and functionality of polarized epithelial cells. It suggests that the polarity of epithelial cells at different anatomic locations of the female reproductive tract alter the mechanisms by which GC establishes an infection (Fig. 21).

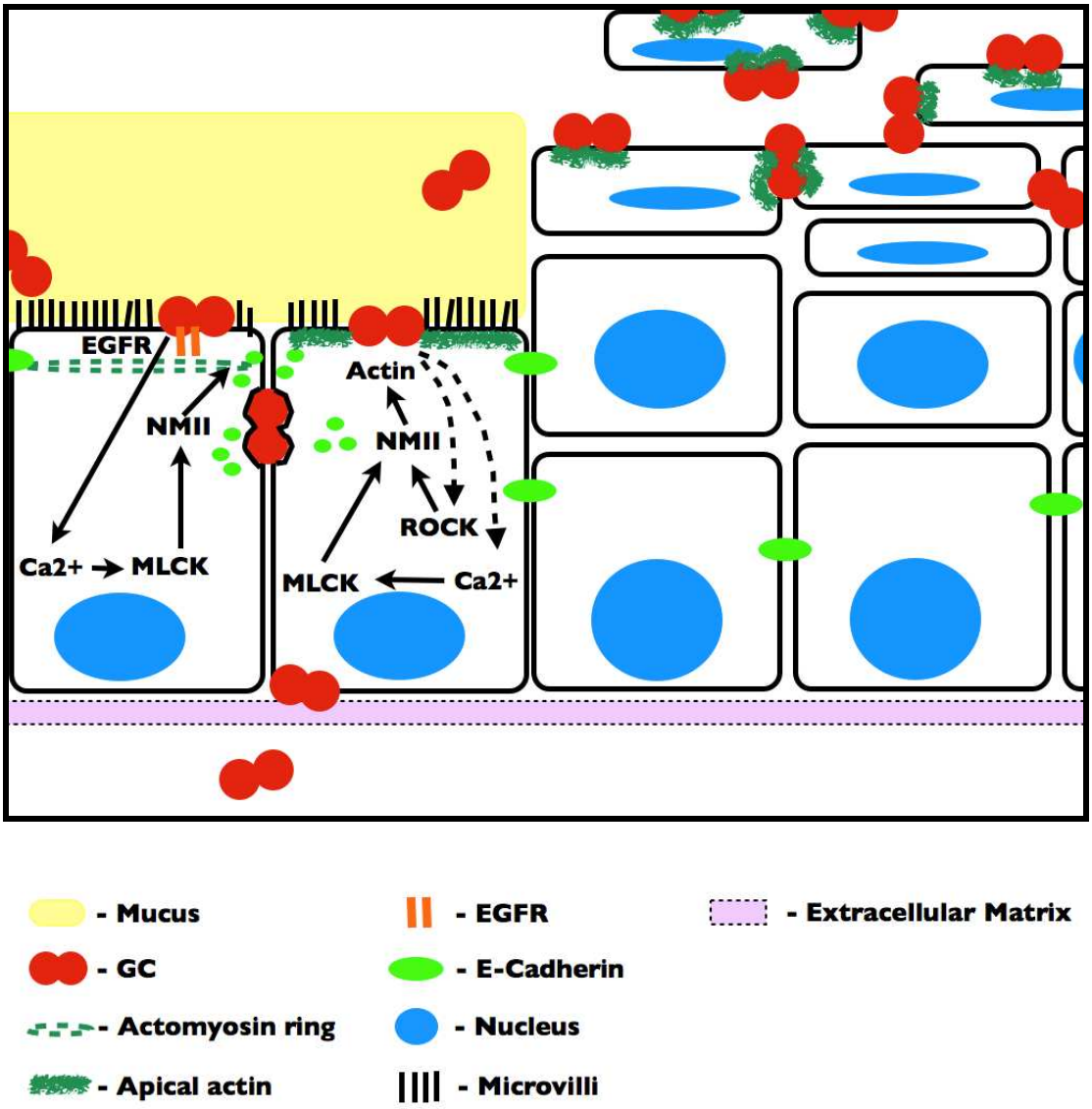

Figure 21. Working model for *Neisseira gonorrhoeae* interaction with epithelial cells along the female reproductive tract.

Infection in endocervical epithelium with polarized epithelial cells (left half) and ectocervical epithelium with non-polarized epithelial cells (right half).

4.2 Working Model

Based on my findings, I propose the following working model. In the ectocervix, there are multilayered non-polarized epithelial cells attached to each other without defined cell-cell junction complexes. During infection, GC (average 7 X 10^6 CFU in semen) spread onto the ectocervical region [144]. GC attaches and adhere to the outmost layer of ectocervix. GC adheres to these cells intimately by inducing actin accumulation underneath their adherent sites and binding to epithelial cell receptors. To ascend to the endocervix and uterus, GC needs to survive in the ectocervical surface. Possible mechanisms for surviving in the ectocervix is to adhere to epithelial surface, invade and escape from the ectocervical epithelial cells, or transmigrate into deeper layer of ectocervical epithelia (Fig. 21). My data suggest that GC adhere to ectocervical epithelium differently from endocervical epithelium. My preliminary data have shown that more GC invades into non-polarized epithelial cells compared with polarized epithelial cells. Previous studies have shown that GC is able to invade into human ectocervical epithelial cells [62] and escape from epithelial cells in culture [31, 35, 145]. Previous studies from our lab have shown that GC microcolonies tend to adhere to the edge of non-polarized epithelial cells, HEC-1-B and ME180 cells, which implicate the possiblility of GC migration between cells into deeper layer. I also have shown that the presence of GC in the space between two adjacent ectocervical epithelial cells (Appendix Fig. 1). It would be difficult for GC to transmigrate through multiple layers of epithelial cells into subepithelial cells in the ectocervix. However, it may allow GC to survive in a changing environment in order to further infect upper reproductive tract. In addition to preventing shedding with the

outer layer of epithelial cells, the ability of GC to enter middle layers of epithelial cells may protect them from interactions with commensal bacteria in the lower reproductive tract that inhibit GC growth. While GC may interact with epithelial cells differently in order to survive the environment, we should be mindful that GC may also phase vary for avoiding antibodies that are secreted/transported by epithelial cells.

In the endocervix, the epithelia lining was a single layer of columnar epithelial cells and some of the epithelial cells are ciliated. The columnar epithelial cells are specialized to secret mucus that has different levels of viscosity and composition depending on the stage in the menstrual cycle. During infection, the number of GC that can reach the endocervix is much lower than that in the ectocervix.
In order to establish infection in endocervical epithelium, GC will need to survive and penetrate the mucus layer for adherence to epithelial cells. GC then can ascend to the endometrial region or cross the single layer columnar endocervical epithelial cells to the subepithelial tissue. To reach utery, GC needs to overcome two obstacles. First, GC has to move through the endocervix that is about 4 cm (1.6 inch) long. Second, endocervical epithelial cells constantly exocytose mucus to the surface of endocervical epithelial cells, which creates difficulties for GC to colonize in a long-term. It is possible that invading into columnar endocervical epithelial cells and subepithelial tissue will allow GC to colonize the reproductive tract region and progress to the upper reproductive track (Fig. 21). The direct contact of GC with the

endocervical epithelial cells in human tissue explants support the notion that GC are able to penetrate the mucus layer and attach to the surface of endocervical epithelium. GC transmigration across the single layer polarized columnar epithelial cells may be essential for GC to survive in the endocervix. There are two possible ways for GC transmigration: intracellular and paracellular pathway. Previous studies have shown GC can invade and escape from epithelial cells [34] However, there is no direct evidence for GC transmigration via paracellular space, even though meningococci (MC) are able to recruit cell junctional proteins to the adherent site and transmigrate paracellularly [146]. Our lab has shown that GC can breach the apical junction by inducing the transactivation of EGFR [124]. My transmission electron microscopic analysis shows the existence of GC in the paracellular space (Appendix Fig. 2). Furthermore, my study demonstrates a close correlation between the magnitude of junction disruption by GC and the efficiency of GC transmigration. Whether and how EGFR, junction proteins, and NMII activation contribute to paracellular transmigration is unknown. The endocervical mucus is an important defense line for the host, as mucus not only inhibits pathogen adherence, but also contain various antibacterial peptides and immunoglobulins [147]. It is unclear whether GC is capable of degrading mucus and/or inhibiting mucus secretion by epithelial cells. My findings that GC can establish adherence with mucus secreting endocervical epithelium support my hypothesis (Fig 8).

Taken together, My Ph.D. research sheds light on the progress of GC infection in the lower female reproductive tract and demonstrates how GC use host cell signaling to

compromise the epithelial barrier and invade into subepithelial tissues. The differential actin remodeling in non-polarized and polarized epithelial cells provides the first evidence supporting the different mechanisms that GC uses for ecto- and endo-cervix infection.

4.3 Future Direction

4.3.1 Highlights

This work examined GC infection in the female reproductive tract at both the broad and mechanistic levels. I compared infection by GC at the ectocervical and endocervical epithelia, and found differences in actin remodeling, which implicates different GC transmigration and invasion mechanisms and contributes to our understanding of GC induced chronic condition and complications. It may also help to develop new strategies for preventing and treating gonococcal infection in the female reproductive tract. I revealed one of the signaling mechanisms by which GC compromise the barrier function of the epithelium and promote their invasion into the polarized epithelium and subepithelial tissue. My studies identify the key host molecules that can be manipulated prevention and treatment of GC infections. Meanwhile, my research has raised many questions and identified many areas that need further research.

4.3.2 Question 1. Does GC elicit different Ca^{2+} signals or does Ca^{2+} signals play different roles in non-polarized and polarized epithelial cells?

This study has shown that GC-induced EGFR dependent Ca^{2+} flux in polarized epithelial cells, which is essential for GC-induced junction disruption and GC transmigration across the epithelium. However, it is not known if GC induces Ca^{2+} flux in non-polarized epithelial cells with the same timing and to the same level, and what is the role for Ca^{2+} signaling in non-polarized epithelial cells. GC has been previously shown to induce transient Ca^{2+} flux in non-polarized epithelial cells, but at 5-20 min post GC infection [9]. The EGFR-dependent activation of Ca^{2+} flux induced by GC in polarized epithelial cells can be detected at 6 hr post GC inoculation. Our lab previously showed that GC induce EGFR transactivation peaking at 4-5 h post inoculation as well as the recruitment of EGFR to GC adherent site in non-polarized epithelial cells. Inhibition of EGFR kinase and Ca^{2+} release from intracellular pool significantly decrease GC invasion in non-polarized epithelial cells [51]. It suggests that EGFR and Ca^{2+} signal have roles in GC infection in non-polarized epithelial cells. However, the relationship between EGFR and Ca^{2+} flux and the relationship between these signaling with actin remodeling are still unknown. It would be interesting to see if the Ca^{2+} flux is induced by EGFR activation in non-polarized epithelial cells and if the signaling pathways are required for actin remodeling and GC invasion. It would be important to know the different calcium signaling pathway(s) in both non-polarized and polarized epithelial cells needed for GC to infect human epithelial cells.

4.3.3 Question 2. How does GC survive in the ectocervical tract on squamous epithelium?

It has been shown that GC can adhere to the ectocervical epithelium. The ectocervical epithelium, as well as the vaginal epithelium, has multiple layers of squamous epithelial cells, where the top layers shed frequently. How GC can colonize and survive in this constantly changing environment in order to ascend to the endocervix has not been addressed. Previous studies have found that GC is inside the shed ectocervical squamous epithelial cells of gonorrheal patients, which suggests GC can invade into ectocervical epithelium [148]. GC has been found in the paracellular space in the epithelium of the female reproductive tract [31]. Our preliminary study (data not shown) has found the presence of GC both intracellularly and paracellularly in the multilayer HEC-1-B cell model much more frequently than in the single layer polarized HEC-1-B epithelial cell. These data lead to the hypothesis that GC survival in ectocervical epithelium depends on not only adherence but also invasion into epithelial cells and into deeper layer of epithelial cells. Future studies should compare GC colonize multiple layers of non-polarized and single layer of polarized epithelial cells.

4.3.4 Question 3. How does GC move across mucus layer to apical epithelium?

I have shown that GC can adhere to the apical surface of the endocervical epithelial monolayer in human cervical tissue explants. This suggests that GC can penetrate the thick layer of mucus that secreted by the endocervical epithelium. How GC can overcome this protective layer to adhere to epithelium is still not known. One hypothesis is that GC can secret a mucinase or enzyme that can degrade mucus as well as associated anti-bacterial peptides and immunoglobulins. So far the only known proteinase is Immunoglobulin A (IgA) proteinase reported by *Hedges* et al. [149], but the IgA proteinase shows no significant activity of mucus degradation. Our preliminary bioinformatics analysis has found numerous putative glycosidase and proteinase in GC genome. Future work should investigate if these putative proteinases and glycosidases have mucus degradation activities. Another possibility is that pili mediate bacterial movement in mucus, and therefore pili's role in GC movement in mucus should also be examined.

The human specific nature and the varying properties of the epithelium along the female reproductive tract are the two major obstacles in GC infection research. My Ph.D. research has established human tissue explants as the models to confirm data generated in cell culture models, which enable us to address the questions that previously were not possible to address. The proposed research will provide a better idea of how GC establish infection at different anatomic locations of the female reproductive tract and therefore provide new directions for preventing gonorrhea and complicated gonorrhea disease.

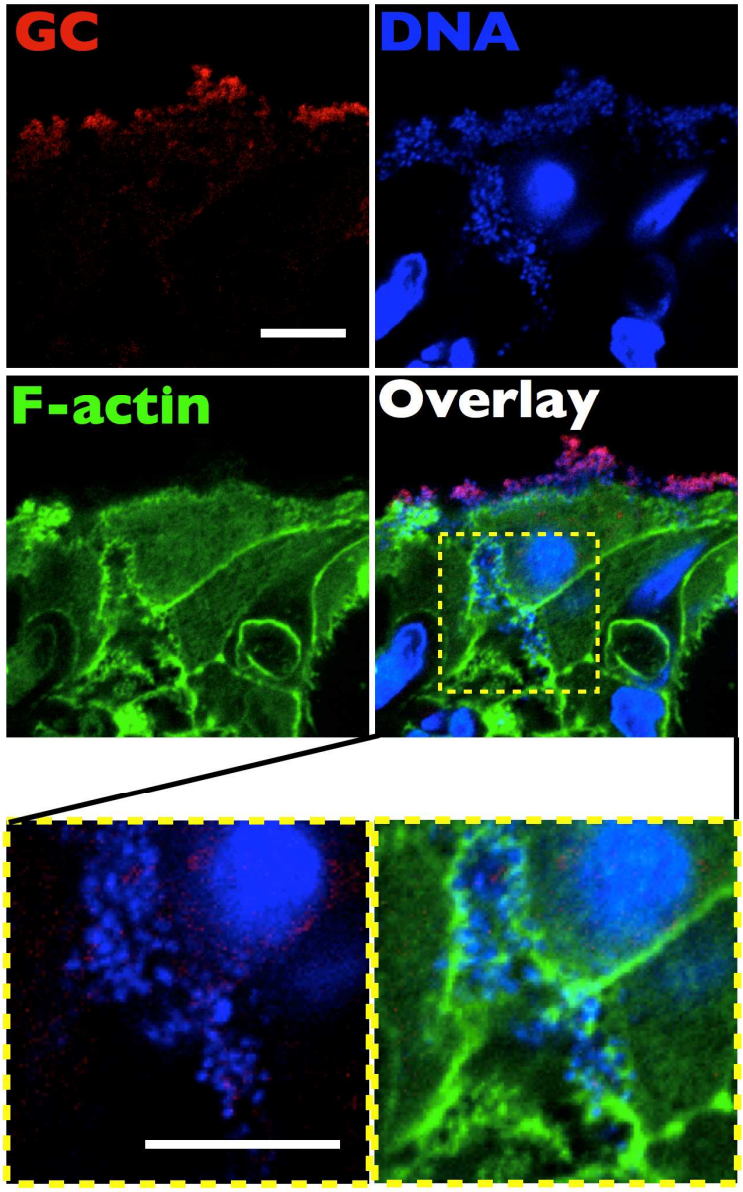

Figure 1. Paracellular GC in ectocervical epithelium

Human ectocervical tissue slides were incubated with wt GC (1x106/ml) for 6 h, washed to remove non-adherent bacteria, and continued the incubation for another 18 h. The tissue was fixed, cryopreserved, sectioned, stained for GC (red), F-actin (green), and DNA (blue), and analyzed using 3D confocal fluorescence microscopy.

Scale bar, 10 μm.

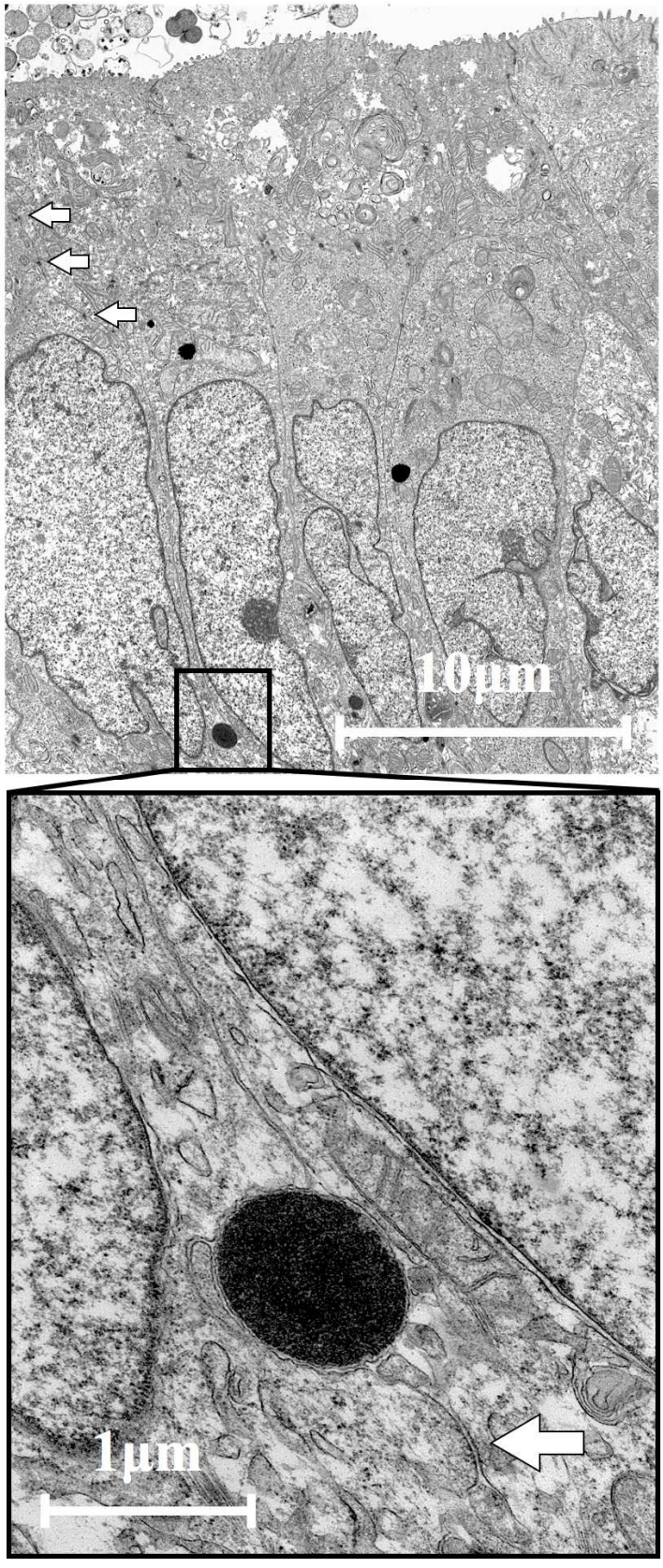
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Figure 2. Electron micrograph of MS11Δopa in the paracellular space.

N. gonorrhoeae MS11Δopa was incubated at a MOI 50 with polarized T84 cells for 24 hr and processed for TEM. Upper panel, Adherens junctions were visualized in condensed area along lateral membrane (arrows). The magnification was 2000X. Bottom panel. GC is visualized between two neighboring cell lateral membranes which can be seen below GC (arrow). The magnification was 16,000X.

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