MicroRNAs cause micro changes: Regulation of expression of membrane-associated complement inhibitors and its effect on *Neisseria gonorrhoeae*

A Thesis Presented to

The Honors Tutorial College

Ohio University

In Partial Fulfillment

of the Requirements for Graduation

from the Honors Tutorial College

with the degree of

Bachelor of Science in Biological Sciences

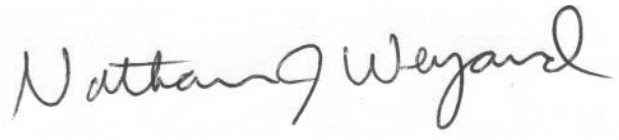
Avital Savin

April 24th, 2021

Thesis Approval Page

This thesis has been approved by

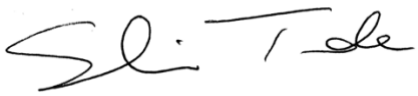
The Honors Tutorial College and the Department of Biological Sciences



Dr. Nathan J. Weyand

Assistant Professor, Biological Sciences

Thesis Advisor



Dr. Soichi Tanda

Director of Studies, Biological Sciences

Dr. Donal Skinner

Dean, Honors Tutorial College

Abstract

Neisseria gonorrhoeae is the causative agent of gonorrhea, the second most common bacterial sexually transmitted infection worldwide following chlamydia. Antibiotic resistance to *N. gonorrhoeae* is growing rapidly, even affecting countries with advanced healthcare systems. *N. gonorrhoeae* has been labeled as an imminent threat due to its high levels of antibiotic resistance. If gonorrhea is left untreated, it can cause sterility, ectopic pregnancy and pelvic inflammatory disease. Many times, *N. gonorrhoeae* is not easily noticed due to its ability to cause asymptomatic infections. Some individuals infected asymptomatically are pharyngeal carriers. Currently, there is no vaccine. Research efforts are focused on vaccine development and other preventative therapeutics.

N. gonorrhoeae may cause infection by attaching to human epithelial cells in the urogenital tract. *N. gonorrhoeae* persistent mechanisms in humans frequently rely on its ability to evade the human immune defenses such as the complement cascade. The aim of this project is to focus on complement regulatory proteins, which are proteins that human cells express to protect cells from autologous complement damage. Our previous data suggests that *N. gonorrhoeae* is stealing complement regulatory proteins, like CD46, CD55, and CD59 to prevent immune activation, allowing the bacteria to persist and cause infection. This project studies the effect of microRNAs, such as miR-200b and miR-200c, on the membrane complement proteins. This was accomplished by transfecting our model cell line, MCF7, with microRNA expression plasmids. The aim of this research is to understand the effect of complement regulatory proteins on *N. gonorrhoeae* survival.

Acknowledgements

This work was in part funded by the Jeanette G. Grasselli Brown Undergraduate Research Award. I am incredibly grateful to the Honors Tutorial College for giving me the opportunity to conduct this proposed study.

Furthermore, I would like to thank the Ohio University Genomic Facility for performing Bioanalyzer and RT-qPCR experiments on my samples.

I am incredibly appreciative for the outstanding mentorship I received from my academic advisor, Dr. Soichi Tanda, throughout my four years at Ohio University. I would also like to sincerely thank Dr. Nathan Weyand, my thesis advisor, for constantly pushing me so that I would grow as a scientist. Dr. Weyand has also provided me with the equipment and materials that were necessary to conduct this research project. I would also like to thank the members of the Weyand Lab (Donnie Pickel, Eliza Thapa, Paul Zdinak, Justin Kellar, and Kinzie Bailey) for their support and help in this research project.

Finally, I would like to thank my family and friends who have encouraged me wholeheartedly throughout my studies.

List of Figures and Tables:

Abbreviations:

1. *N. gonorrhoeae*	*Neisseria gonorrhoeae*
2. mCI	Membrane-associated complement inhibitor
3. PBP	Penicillin Binding Protein
4. CMRNG	Chromosomally Resistant *N. gonorrhoeae*
5. PPNG	Penicillinase Producing *N. gonorrhoeae*
6. MAC	Membrane Attack Complex
7. CD46	Membrane co-factor protein
8. CD55	Complement decay-accelerating factor (DAF)
9. CD59	Protectin
10. C3	Complement component 3
11. C5	Complement component 5
12. C9	Complement component 9
13. mRNA	Messenger RNA
14. miR	MicroRNA
15. RNP	Ribonucleoprotein
16. RISC	RNA-Induced Silencing Complex
17. AGO	Argonaute protein
18. UTR	Untranslated Region
19. ARE	AU-Rich Elements
20. FXR1	Fragile-X mental-retardation-related protein 1
21. MCF7	Michigan Cancer Foundation-7
22. DMEM	Dulbecco's Modified Eagle Medium

23. FBS	Fetal bovine serum
24. MS11	Wild-type *N. gonorrhoeae*
25. cDNA	Complementary DNA
26. RIN	RNA Integrity Number
27. RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
28. PBS	Phosphate-buffer saline
29. FITC	Fluorescein isothiocyanate
30. APC	Allophycocyanin
31. PE	Phycoerythrin
32. FMO	Fluorescence minus one
33. MFI	Mean Fluorescence Intensity
34. ccSBA	Co-culture serum bacterial assay
35. OD	Optical Density
36. NHS	Normal Human Serum
37. ns	No significance
38. HBXIP	Hepatitis B X-interaction protein

Table of Contents:

Introduction

This thesis will describe some of the characteristics of *Neisseria gonorrhoeae* which causes the well-known infection gonorrhea. The subject of gonorrhea is important for biologists and doctors to study because of antibiotic resistance, a function that many different bacteria are rapidly gaining. Antibiotic resistance is threatening the ability to treat many different infections. As researchers work to develop novel therapeutics to treat these types of infections, it helps them immensely if they know the mechanisms bacteria use to cause infection. This thesis will review one of the ways that *N. gonorrhoeae* is able to persist during infection by evading the immune system. Specifically, the study will explore how certain proteins on the surface of human cells may affect the survivability of *N. gonorrhoeae* during infection of human cells. The production of these proteins is tightly regulated by multiple factors. These protein's genes can be upregulated or downregulated, meaning that their expression in the cell can be increased or decreased. A well-researched class of molecules, known as microRNAs, typically downregulate gene expression, but can also upregulate gene expression in some cases. Although there are multiple methods used to upregulate gene expression, microRNAs will be used as tools to alter expression of genes that encode a class of protective human proteins called membrane-associated complement inhibitors (mCIs). mCIs are hypothesized to be utilized by *N. gonorrhoeae* during infections of human epithelial cells to evade innate immunity. Specific microRNAs reported to target mCI genes and upregulate or downregulate their production will be evaluated. MicroRNAs that potentially upregulate mCIs have been selected.

Currently, human cells that express altered mCI levels are being generated. Human cell culture infection experiments were used to study if altering microRNA expression influences mCI expression and survival of *N. gonorrhoeae* during infection. The hypothesis of this study is that microRNA-dependent increases in mCIs expression will increase survival of *N. gonorrhoeae.* Furthermore, microRNAs that decrease the expression of mCIs will have the opposite effect. If these microRNAs alter of survival of *N. gonorrhoeae*, then they may serve as therapeutic targets capable of sensitizing *N. gonorrhoeae* to innate immune defenses against a *N. gonorrhoeae* infection.

Neisseria gonorrhoeae

The genus *Neisseria* consists of at least 23 species, two of which are pathogenic to humans[2]. *N. gonorrhoeae,* one of these pathogenic species, is the causative agent of gonorrhea, the second most common bacterial sexually transmitted infection worldwide, following chlamydia[3]. The Centers for Disease Control and Prevention (CDC) states that antibiotic resistance in *N. gonorrhoeae* is growing rapidly, even affecting countries with advanced healthcare systems. Antibiotic resistance allows bacterial survival during treatment with the drugs previously efficacious against gonorrhea[4]. The CDC (2019) published a report labeling *N. gonorrhoeae* as an urgent threat due to its high levels of antibiotic resistance[5]. In many cases, after bacteria become resistant to an antibiotic, different methods need to be taken in order to treat the infection[4]. The cocktail of antibiotics that is currently given to infected patients is an injectable shot of ceftriaxone with oral azithromycin, though there have been cases in which *N. gonorrhoeae* is resistant to this treatment[6,7]. Figure 1 depicts the history of antibiotic resistance in *N.*

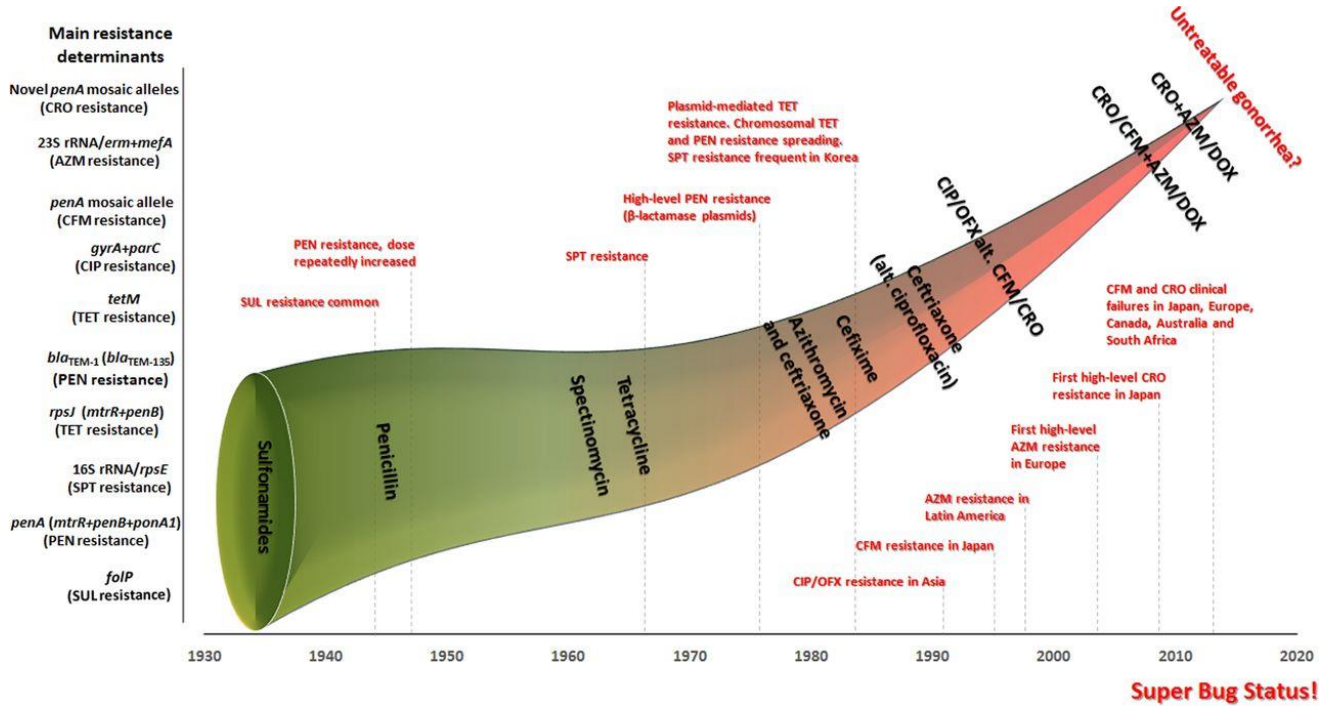


Figure 1. The history of antibiotic resistance in *N. gonorrhoeae*[8].

gonorrhoeae. Therefore, alternative therapies are needed to avoid entering an era of untreatable gonorrhea infections. If gonorrhea is left untreated, it can cause sterility, ectopic pregnancy, and pelvic inflammatory disease[5,9]. It can also increase the chance of contracting or transmitting HIV infections[5]. *N. gonorrhoeae* frequently persists undetected in human population because of its ability to cause asymptomatic infections. Some individuals infected asymptomatically are pharyngeal carriers, meaning their infection resides in the throat[9]. This means that *N. gonorrhoeae* may be transmitted from person to person not only through penetrative sex but also through oral sex.

Due to the infection being largely asymptomatic, this increases the chance of passing along the infection. *N. gonorrhoeae* has a high rate of infection, with more than 1.14 million cases of gonorrhea in the US alone. 550,000 of these infections are drug-

resistant[5]. The World Health Organization (WHO) also states that 78 million people worldwide are infected with *N. gonorrhoeae* every year[9]. Currently there is no vaccine. Research efforts are focused on developing vaccines, antibiotics and other preventative therapeutics.

Antibiotic Treatments

N. gonorrhoeae has been treated with many different antibiotics throughout history (Fig. 1). These various treatments have aided *N. gonorrhoeae* in its antibiotic resistance ability. Many antibiotics target the peptidoglycan wall of the bacteria[10]. Peptidoglycan is essential for a bacterium to have a cell wall. Specifically, the penicillin binding proteins (PBPs) that *N. gonorrhoeae* has are the main targets of these antibiotics[10]. The two PBP enzymes found in *N. gonorrhoeae* are class A PBP1 and class B PBP2[10]. PBPs are essential enzymes that contribute to peptidoglycan biosynthesis. After scientists observed the killing of bacteria due to penicillin, they discovered the PBPs. β-lactam antibiotics are a class of antibiotics that possess a β-lactam ring which include penicillin and all derivatives, cephalosporins, and monobactams. Ampicillin and amoxycillin are derivatives of penicillin[11]. These antibiotics target PBPs and reduce the ability for the bacteria to build a cell wall. PBPs bind these antibiotics due to the similar chemical structure to peptidoglycan. There are a few different reasons that *N. gonorrhoeae* is able to resist antibiotic treatment. Alterations to the gene encoding PBP2, known as *penA*, reduces the binding affinity of the PBP to the β-lactam antibiotics[10,12]. Other loci can also acquire mutations resistant to antibiotics. A mutation at the *mtr* locus caused resistance through an active efflux system and a mutation at the *penB* locus

reduced permeability of the cell envelope to antibiotics[11]. The bacteria containing *mtr*, *penA,* and *penB* mutations are known as chromosomally resistant *N. gonorrhoeae* (CMRNG). Along with alterations in the loci of the bacterium, resistance to penicillin and its derivatives occurred due to inducible TEM-1 type β-lactamase[11]. This enzyme is capable of hydrolyzing the β-lactam ring. The bacteria containing this enzyme were called penicillinase producing *N. gonorrhoeae* (PPNG). As a result, *N. gonorrhoeae* became resistant to the β-lactam antibiotics. The antibiotic resistant mechanisms possessed by *N. gonorrhoeae* against penicillin were also affective against cephalosporin, spectinomycin and aminoglycosides. Some studies have shown that spectinomycin resistance is due to a mutation in the ribosomal protein S5[13,14]. Transformation, which is the transfer of genetic material to a bacterium by intake of exogenous genetic material from the environment, is used by *N. gonorrhoeae*[15]. Through this ability, antibiotic resistance is widespread.

Complement Inhibitors

N. gonorrhoeae may cause infection by attaching to human epithelial cells in the urogenital tract[16,17]. Epithelial cells are located on mucosal surfaces and serve as a protective barrier between the inside and outside of the body. *N. gonorrhoeae* persistence mechanisms include the evasion of human immune defenses such as the complement cascade[17]. The complement system can recognize foreign invaders such as bacteria and target them for destruction. Complement system proteins attach to bacterial membranes and form a structure called the membrane attack complex (MAC). The MAC forms holes in bacterial cells causing them to burst and die. Because complement proteins can also

attach to human cells and damage their membranes, cells in the human body express membrane-associated complement inhibitors (mCIs) to prevent MAC-dependent damage[18]. Consequently, mCIs serve as protectors of the cell. Figure 2 depicts how CD46, CD55, and CD59 inhibit the complement cascade and protect the cell from attack by the immune system.

These mCIs are located on the cell surface of human epithelial cells. CD46, which is a Type 1 membrane protein, is also known as Membrane Cofactor Protein. CD55 is also known as Decay-accelerating factor (DAF). CD59 is also known as protectin. CD55 and CD59 are glycosylphosphatidylinositol anchored (GPI-anchored) proteins. All three mCIs bind to the phospholipid bilayer of the cells.

Complement component 3, also known as C3, is a vital protein in MAC formation because its cleavage marks cells for death. C3 is used in all three complement pathways: classical, lectin, and alternative. For C3 to be activated, C3-convertases cleave C3 into C3b and C3a. C3b is able to combine with other proteins to form another enzyme called C5-convertase which cleaves complement component 5 (C5). Ultimately, C5 recruits proteins to form the MAC. mCIs expressed on human cells inhibit different parts of the complement pathway as shown in Figure 2 to avoid cell death. CD46 and CD55 both promote inactivation of C3b and C4b through cleavage[19,20]. Both of these complement components are used to form C3-convertases and C5-convertases. CD59 inhibits the MAC by preventing complement component 9 (C9) from polymerizing[21]. Polymerization is the binding of a protein to other proteins, in this case C9 binds to itself. The polymerization of C9 forms the pore-forming unit of the MAC. These three mCIs are used by cells to inhibit the complement cascade which causes cell death.

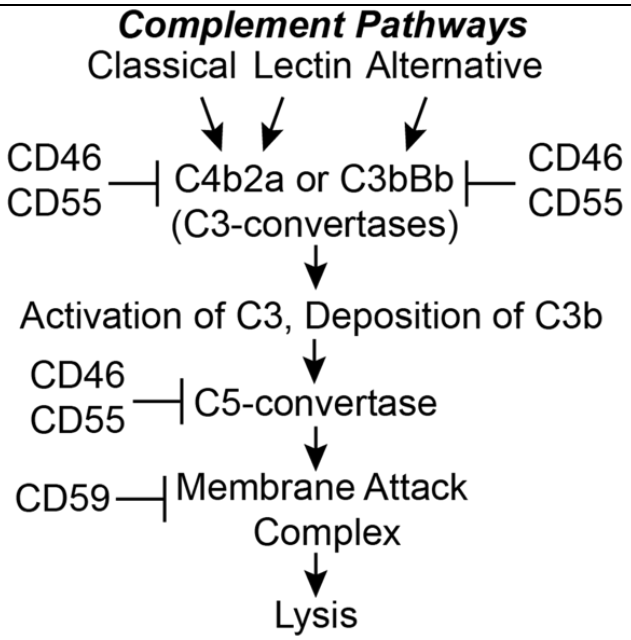


Figure 2. The complement pathway involves mCIs such as CD46 and CD55 to inhibit the complement cascade.

Multiple labs have shown mCIs are recruited by *N. gonorrhoeae* during infection of human cells[22,23,24,25]. Figure 3 (unpublished data, Weyand et al.) shows an immunofluorescent image of CD46 clustering around the human cell. The image exhibits that the mCIs are not only on the surface of the bacterial microcolony, but also imbedded within the microcolony. This is shown by the XZ and YZ planes. The hypothesis of this study is that *N. gonorrhoeae* is stealing mCIs from infected cells to protect bacterial membranes from MAC-dependent killing.

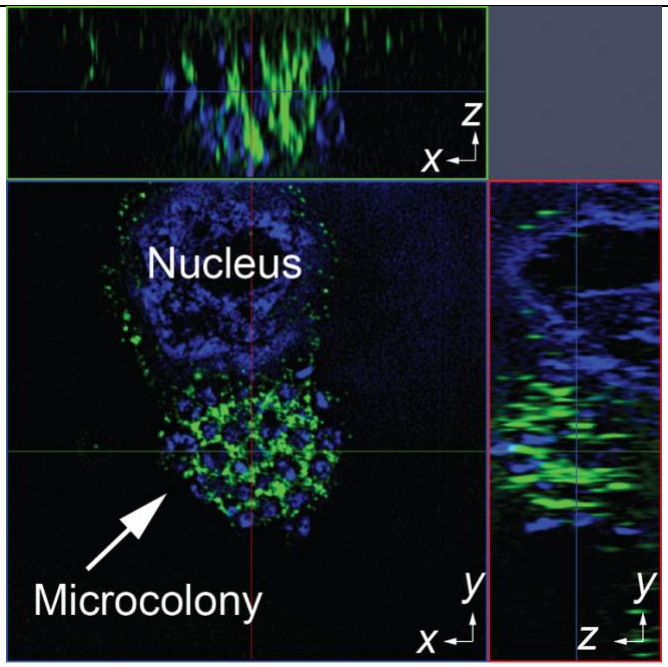


Figure 3. Immunofluorescent staining of CD46 in End1 cells and *N. gonorrhoeae.* The nucleus of the human epithelial cell and the nucleus of *N. gonorrhoeae* are stained blue by DAPI. CD46 is stained green. CD46 is clustering around the bacterial microcolony three hours post-infection.

MicroRNAs

The three mCIs that this research project will focus on are CD46, CD55, and CD59. These mCIs can have varying protein expression levels in different tissues. Their expression levels can be manipulated so that they are upregulated or downregulated in the human cells[1]. The central dogma of molecular biology states that DNA is transcribed into RNA which is then translated into protein[26]. Protein coding RNAs are called messenger RNAs (mRNAs). MicroRNAs (miRNAs) are very short non-coding RNAs that regulate gene expression post-transcriptionally[27]. Specific microRNAs are expressed in different tissue types in humans[28]. Approximately 2,600 different microRNAs are encoded by the

human genome[29]. Non-coding RNAs do not get translated by ribosomes to produce a protein. MicroRNAs frequently downregulate gene expression by binding to certain regions of mRNAs to stimulate their degradation[30]. When microRNAs stimulate mRNA degradation, they reduce the production that would normally occur when the targeted mRNA was translated. MicroRNAs can also directly inhibit translation by forming complexes with mRNAs.

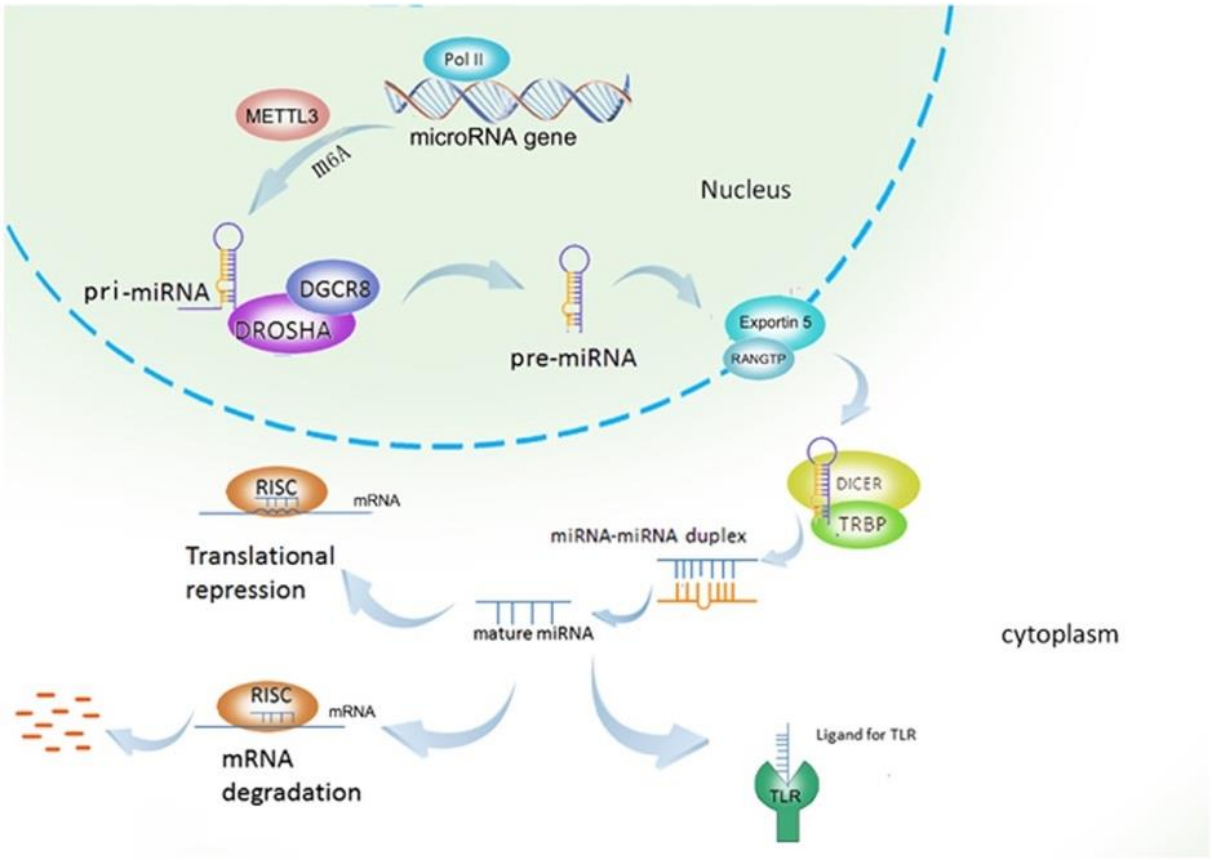


Figure 4. Mechanisms used by microRNAs to downregulate gene expression[30].

While microRNAs typically downregulate gene expression, there are examples of microRNAs that upregulate gene expression[27,30–42]. Figure 4 depicts a mechanism used by

microRNAs to downregulate gene expression[30]. For downregulation of gene expression, microRNAs recruit ribonucleoprotein (RNP) complex to the complementary RNA[43]. This microRNA-RNP complex is called the RNA-induced silencing complex (RISC). The Argonaute protein (AGO) is part of the RNP that binds the microRNA[44]. The RISC complex binds to the mRNA to initiate translation repression. This is a method by which many microRNAs downregulate gene expression. As mentioned previously, microRNAs can also upregulate gene expression. Vasudevan et al. showed that upregulation of TNFα expression through microRNP-associated proteins occurs in mRNAs 3' untranslated regions (UTRs)[36,45].

This means that proteins that can associate with microRNAs to decrease expression can also play a role in upregulation of TNFα. AU-rich elements (AREs) present in the 3' UTR of mRNAs can associate with microRNAs[33,34,45,46]. The AREs, which can effect changes in the translation of mRNA, associate with proteins fragile-X mental-retardation-related protein 1 (FXR1) and Argonaute 2 (AGO2) during translation activation. The binding of AGO2 and FXR1 upregulated expression of TNFα in the study conducted by Vasudevan et al[45]. Although many studies have shown upregulation of protein expression due to microRNAs, the specific mechanism is not yet known. The targets of many microRNAs can be found using different databases like Target Scan and DIANA Tools[47,48]. There has been some research conducted on the upregulation of mCIs CD46 and CD55 by three microRNAs called miR-200b, miR-200c, and miR-217[1]. Although these microRNAs were shown to upregulate CD46 and CD55, the method by which this occurs is unknown. The literature has also shown that these microRNAs do not have any effect on the protein expression of CD59[1].

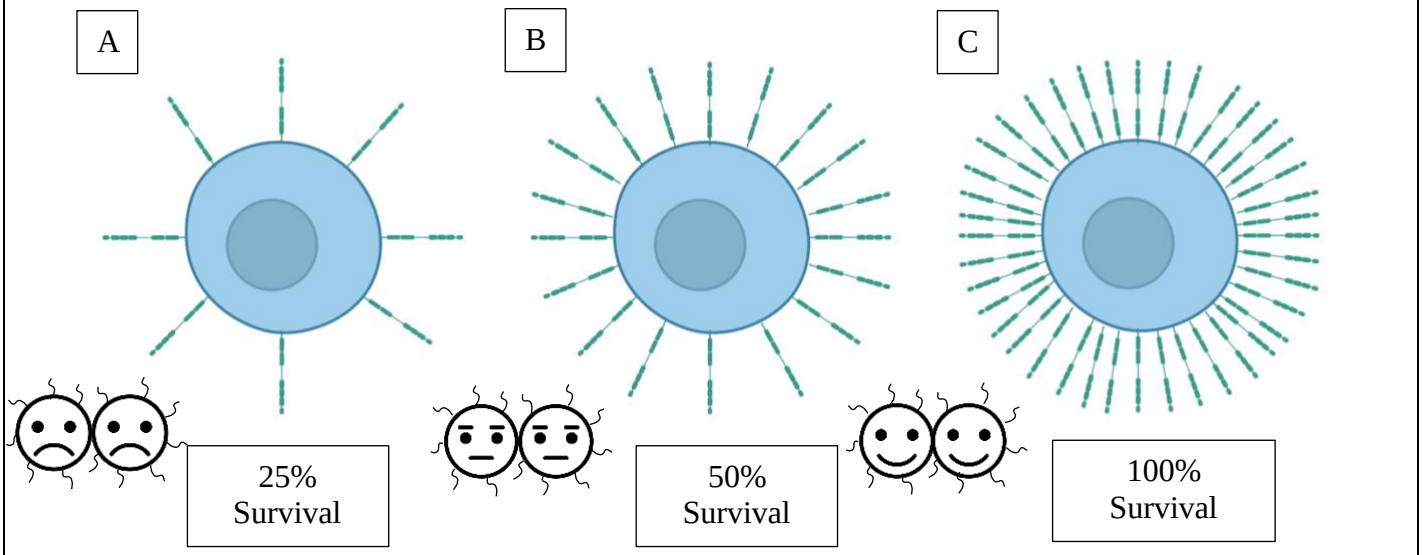


Figure 5. This figure shows the predicted survivability of *N. gonorrhoeae* during infection of human cells that are expressing different amounts of mCIs. The green receptors represent the mCIs on the blue human epithelial cells. The different faces represent the survivability of *N. gonorrhoeae*. Panel A shows a human cell with low amounts of mCI. This type of human cell is predicted to support lower *N. gonorrhoeae* survival because it has less mCI surface expression. Panel B is a human cell that is expressing normal amounts of mCI. Panel C shows a human cell with many mCIs which are predicted to support increased survival of *N. gonorrhoeae.* The text boxes below each image indicate hypothetical percent survival levels of *N. gonorrhoeae.* Created with BioRender.com

The literature has suggested that *N. gonorrhoeae* may steal mCIs from human cells during infection to evade complement killing by the MAC. It is hypothesized that mCIs CD46, CD55, and CD59 are utilized by *N. gonorrhoeae* to protect bacterial membranes from complement-dependent killing. It is unknown which of these mCIs are the most responsible for the survival of *N. gonorrhoeae.* Figure 5 is a cartoon illustration of our model predicting the effect of different mCIs expression levels on *N. gonorrhoeae* survival in the presence of complement proteins.

Our experiments will attempt to manipulate mCI expression levels, so that they are either upregulated or downregulated. These manipulations will be carried out using microRNAs that target the genes for mCIs CD46 and CD55. Initially, the focus of this study will be on an approach used by Hillman et al. that used microRNAs to upregulate the expression of CD46 and CD55 in human cells[1]. The study conducted by Hillman et al. showed that miR-200b and miR-200c had no effect on the protein expression levels of CD59[1]. Figure 6 shows the results obtained by Hillman et al[1]. The figure combines their results with the complement cascade shown in Figure 2. The data shows that miR-200b and miR-200c both upregulated synthesis of C46 and CD55. These microRNAs did not affect the synthesis of CD59. With increased expression of miR-200b and miR-200c, Hillman et al. also observed decreased activity of C3 activation and MAC formation.

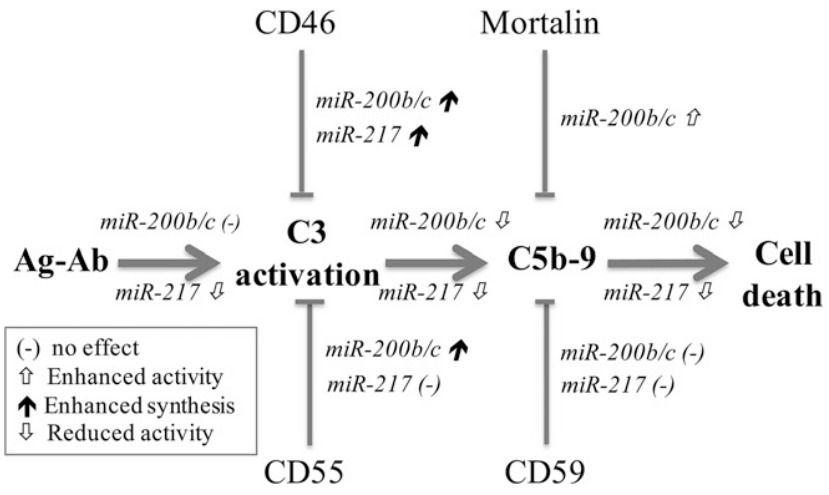


Figure 6. Data obtained by Hillman et al. regarding the effect of different microRNAs on expression of mCIs[1]. miR-200b and miR-200c were shown to increase expression of CD46 and CD55. Neither microRNA increased synthesis of CD59. The black bold arrows show which microRNA increased synthesis of which protein. C5b-9 is also known at the MAC.

The purpose of this study is to explore different microRNAs and their effects on gene expression of mCIs such as CD46, CD55, and CD59. If mCIs are used by *N. gonorrhoeae* to evade the human immune system, then a therapeutic other than antibiotics can be developed to minimize infectivity of *N. gonorrhoeae.* There are many health risks associated with *N. gonorrhoeae* and the continued rise of antibiotic-resistance complicates treatment. Therefore, a new therapeutic must be developed soon. Other targets for therapeutics must be found in order to avoid untreatable *N. gonorrhoeae* infections. These targets may include microRNAs, mCIs or a combination of both.

MCF7 Cells

This study will be primarily using MCF7 cells as a model. MCF7 cells, abbreviated from Michigan Cancer Foundation-7 cells, are an epithelial breast cancer cell line[49,50]. These cells were isolated in 1970 from a 69-year old Caucasian woman in Detroit, Michigan by Herbert Soule[49]. Therefore, these cells are human cells. They were isolated from the metastatic site in the mammary gland of the female breast[51]. MCF7 cells are a very popular choice in conducting research because they were the first mammary cell line that was capable of living longer than a few months[52]. Therefore, these cells significantly impacted the ability to research breast cancer. Along with their stability, MCF7 cells have many characteristics that have been beneficial to studying breast cancer.

Although MCF7 cells are typically used for studying breast cancer related topics, they are also a good model for studying mCIs. MCF7 cells have limited expression of

mCIs compared to other types of cells[53,54]. Alternatively, mCI levels can be easily manipulated in MCF7 cells[55]. This can also be demonstrated by the data shown in Figure 7 (unpublished data, Weyand et al.). mCI surface expression in other cell types, like human endocervical ME180 cells is much higher[25]. If cells have lower amounts of expression, that means that when the expression levels are manipulated, either upregulated or downregulated, the effect is easier to detect. In this study, MCF7 cells were used to study the effect of miRNAs on protein expression of multiple mCIs.

ME180 Cells

ME180 cells are human endocervical cells isolated from epidermoid carcinoma[56]. This cell like has been a common model for *N. gonorrhoeae* infections[16,22,24,25]. Preliminary attempts to manipulate mCI expression levels did not result in *N. gonorrhoeae* survival phenotypes during infection assays. Therefore, we chose to develop a model system using MCF7 cells.

Preliminary Results

It is believed that *N. gonorrhoeae* survival is dependent on mCI expression due to data shown in Figure 7 (unpublished data, Weyand et al.). The data shows that MCF7 cells, which have a lower amount of CD46 present, support a lower level of *N. gonorrhoeae* survival compared to ME180 endocervical cells. This data suggests that the ability of *N. gonorrhoeae* to survive may be affected by mCI expression. First, microRNAs that associate with mCIs and regulated mCI expression were identified. Although many different microRNAs are predicted to target CD46, CD55, and CD59, we

focused on the ones that have been studied previously. Table 1 shows some of the microRNAs that have been shown to regulate the expression of mCIs.

Table 1. MicroRNAs that have been shown to target genes that encode mCIs and influence their RNA and Protein expression

MicroRNAs	Source suggesting microRNA targets mCI	Expression of mCIs by microRNA
miR-200b/c and miR-217	Hillman et al.[1]	↑
miR-520b and miR-520e	Cui et al.[57]	↓
miR-19a and miR-20a	Tan et al[58]	↓

Although Figure 7 only shows a Western Blot for the expression of CD46, we hypothesize that the other mCIs, CD55 and CD59, also have lower expression in MCF7 cells compared to the ME180 cells. The collective expression of all three mCIs may play a role in the survivability of *N. gonorrhoeae.* This preliminary data was fundamental to the development of our hypothesis that mCI levels may influence *N. gonorrhoeae* survival during infection.

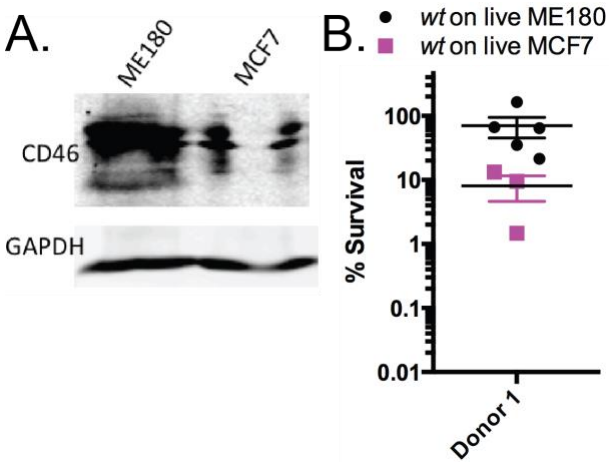


Figure 7. A) This panel shows Western Blot data which measures the amount of CD46 present in two cell lines. The MCF7 cells have less CD46 than ME180 cells. B) This graph shows the percent survival of *N. gonorrhoeae* after a five-hour epithelial infection and serum treatment. *N. gonorrhoeae* survival is lower in MCF7 cells than in ME180s. (unpublished data, Weyand et al.)

Research Question and Approach

This study will focus on several microRNAs predicted to influence mCI expression in human cells. MicroRNAs will be used as tools to upregulate expression of the mCIs CD46 and CD55 in human cells. mCI expression changes will be monitored and correlated with survivability of *N. gonorrhoeae* during infection of epithelial cells. It is predicted that the results of the data collected will mimic that in Figure 5. MicroRNAs that were mentioned in a study conducted by Hillman et al. will be assessed. Hillman et al. used K562, Raji, and HEK293T cells to study to effects of miR-200b, miR-200c, and miR-217[1]. K562, Raji, and HEK293T cells are lymphoblasts, B-lymphocytes, and embryonic kidney cells[59–61]. We will be using MCF7 cells instead. The purpose of the study conducted by Hillman et al. was to show that these microRNAs regulate mortalin expression. Mortalin is a chaperone protein located in the mitochondria. It is a member of

the heat shock protein 70 (Hsp70) family. Mortalin has numerous functions in the cell including inhibition of apoptosis and regulation of proliferation[62]. We will be using the microRNAs as a method to increase mCI expression in cells and testing the survivability of *N. gonorrhoeae* after infection of MCF7 cells. Table 1 shows microRNAs that were used in other studies to manipulate expression of mCIs. If the specific microRNAs mentioned by Hillman et al. upregulate expression of CD46 and CD55 in the model system used by this study, it is hypothesized that *N. gonorrhoeae* will have a higher rate of survival due to the abundance of these proteins[1].

Target Scan and DIANA tools were utilized to find more microRNAs that downregulate CD46 and CD55 if needed[47,48]. The research conducted in this study targets human genes to observe if the bacteria will become more susceptible or more resistant to killing by complement immune defenses. It is also hypothesized that decreasing or increasing the pool of mCIs available for *N. gonorrhoeae* to steal from human epithelial cells will affect bacterial survival in human serum, a source of complement. These microRNAs may indirectly affect the survivability of *N. gonorrhoeae* by influencing mCI expression. The aim of this research is to create cell lines with altered microRNA levels that lead to altered mCI expression levels. Next, we will study the survival of *N. gonorrhoeae* during infection of the newly created cell lines in the presence of complement. This is an important topic to study because it may contribute to our knowledge about host factors used by *N. gonorrhoeae* during evasion of complement killing. Such knowledge could inform the future development of new therapeutics. Such therapeutics would be alternatives to antibiotics, that make *N. gonorrhoeae* more sensitive to the body's natural immune defense system of complement. Few studies have

been done on microRNAs and *N. gonorrhoeae.* Because of the lack of information on upregulation from microRNAs, there are no papers available that link the effect of the microRNAs in human epithelial cells and *N. gonorrhoeae* survival. To accomplish these goals, the following goals will be pursued.

Research Goals

Goal 1: Identify microRNAs which increase or decrease CD46, CD55, and CD59 expression in MCF7 cells

We will be using microRNAs that have shown alteration in gene expression of CD46 and CD55. Hillman et al., Cui et al., and Tan et al. have successfully altered gene expression of the mCIs in question[1,57,58]. Using Target Scan and DIANA Tools, which are databases that predict targets of microRNAs, we searched for other microRNAs that target these mCIs[47,48]. Attempts to identify microRNAs that influence mCIs' expression will inform future efforts like ours to create cell lines that overexpress these microRNAs.

Goal 2: Transfect MCF7 cells

Once we have identified the microRNAs which potentially increase CD46, CD55, and CD59, we will acquire or create plasmids that express the specific microRNAs. We will manipulate plasmids as needed to express the target microRNAs. Plasmids expressing target microRNAs will be transfected into MCF7 cells.

Goal 3: Sequence the Plasmid

We will use Sanger sequencing to confirm the plasmid includes the target microRNA. We will compare the plasmid sequence to the NCBI sequence for each microRNA.

Goal 4: Confirming the microRNA effect on mCIs

Once Goal 3 is complete, we will determine if the microRNA levels and mCI levels changed. We will use RT-qPCR to detect the presence of the microRNAs within the MCF7 cells. Flow cytometry will also be performed to detect the upregulation or downregulation of the mCIs. Specifically, RT-qPCR will detect the transcript levels of the proteins and microRNAs while flow cytometry will detect the surface expression of mCIs.

Goal 5: Testing the effect of microRNA expression on *N. gonorrhoeae* survival

We will test the survival of *N. gonorrhoeae* in a co-culture serum bacterial assay using the microRNA expressing cell lines. We hope to show a correlation of mCI levels and *N. gonorrhoeae* survival. MCF7 WT cells will be the negative control group.

Experimental Design

A previous study done by Hillman et al. has shown upregulation of CD46 and CD55 by miR-200b/c and miR-217[1]. The plasmids containing miR-200b and miR-200c will be stably transfected into MCF7 breast cancer cells, so that the cells will express higher microRNA levels. MCF7 cells will be used because their mCI expression is easily

manipulated in lab settings. Once the plasmids are inserted into the cells, flow cytometry and RT-qPCR will be used to monitor mCI expression. These techniques will attempt to confirm if the microRNAs influence mCI expression in MCF7 cells. Specifically, flow cytometry will measure mCI protein surface levels of cells while RT-qPCR will measure changes in RNA levels for each mCI and microRNA. Before the cells are able to be tested for the microRNAs, they must undergo selection. Selection occurs by allowing the cells to grow in media that has an antibiotic (e.g., puromycin). The plasmids that are inserted into the cells encode a puromycin resistance cassette. Only cells that have taken up the plasmid will survive when put into media containing this specific antibiotic. Once the cells have undergone selection, we will conduct RT-qPCR to quantitatively analyze the presence of the microRNAs in the cells compared to the negative control cell line, MCF7 WT. When we obtain data that verifies the higher presence of the respective microRNA in each cell line, we will conduct flow cytometry. This will be done to observe if there is an increase or decrease in CD46, CD55, and CD59 on the surface of each mutated cell line compared to the negative control cell line. This experiment will quantify the expression of the mCIs in each cell line. We hypothesize that overexpression of miR-200b and miR-200c in MCF7 cells will alter mCI expression as presented in Table 2 (see also the Anticipated Outcomes section below). We have identified microRNAs reported to upregulate and downregulate mCI expression. Table 1 shows the microRNAs that we are most interested in. We will derive miR-200b and miR-200c plasmids and inserted them into MCF7 cells. After we record the difference in expression, we will conduct a survivability assay.

The cell lines developed in Goal 1 with altered mCI levels will be used in a bacterial survival assay. A survival assay tests the ability of *N. gonorrhoeae* to survive during infection of MCF7 cells in the presence of complement proteins. The overexpressed-microRNA MCF7 cells will grow in a dish until they almost cover the whole dish. Then the cells will be infected with *N. gonorrhoeae* for 5 hours. Once the cells are infected, human serum (a source of complement proteins) will be added to the dish which allows the MAC to assemble on bacterial cell membranes. As mentioned earlier, the MAC punctures holes in the cell. We will be studying the influence of microRNA-dependent mCI upregulation and downregulation on *N. gonorrhoeae* survival in serum killing assays during MCF7 cell infections. Serum killing occurs because of the complement proteins found in serum. The infected cells will be processed to count viable bacteria using a Standard Plate Count. Agar plates are used to grow bacteria, so these plates allow us to enumerate the number of live bacteria that were present during infection. This assay will determine if there is an increase or decrease in survivability of *N. gonorrhoeae*. A statistical analysis will be performed on the results of this assay to determine if the MCF7 cells that were expressing microRNAs influence the survivability of *N. gonorrhoeae* as predicted in Figure 5 and Table 2. The result of these experiments will determine if levels of *N. gonorrhoeae* survival in the survival assay correlate with increases or decreases of mCI expression.

Anticipated Outcomes

It is predicted that the microRNAs will affect the survivability of *N. gonorrhoeae* during infection of MCF7 cells exposed to complement if they alter mCI expression.

Table 2 shows microRNAs and their anticipated effect on expression levels of mCIs. The effect of the overexpressed microRNAs is compared to the negative control MCF7 WT cells. MicroRNAs like miR-200b/c and miR-217 are predicted to increase expression of mCIs. If miR-200b and miR-200c overexpression in MCF7 cells increases mCI expression, we predict *N. gonorrhoeae* will survive better in these cells than during infection of the parent control cell line, MCF7 WT. MicroRNAs like miR-520b and miR-520e have been shown to decrease expression of mCIs. If miR-520b/e overexpression in MCF7 cells decreases mCI expression, it is hypothesized that *N. gonorrhoeae* will have lower or decreased survival compared to infection of parent control cells.

Table 2. Predicted effects of microRNA expression on mCI protein expression and *N. gonorrhoeae* survival

MicroRNA expressed in MCF7 cells	**CD46 Expression**	**CD55 Expression**	**Bacterial survival**
MCF7 WT cells (negative control)	Normal	Normal	Basal (normal)
miR-200b/c	Increase	Increase	Increase
miR-217	Increase	Increase	Increase
miR-520b	Decrease	Decrease	Decrease
miR-520e	Decrease	Decrease	Decrease
miR-19a	Decrease	Decrease	Decrease
miR-20a	Decrease	Decrease	Decrease

As stated earlier, it is hypothesized that the microRNAs that increase the expression of CD46 and CD55 will increase survival of *N. gonorrhoeae* and the microRNAs that decrease the expression of CD46 and CD55 will have the opposite effect. The experiments that will be conducted could support the idea that mCIs might serve as a therapeutic target for future treatments of *N. gonorrhoeae* infections. Specifically, the microRNAs can become targets of treatment. If the microRNAs that downregulate mCI expression also reduce the survivability of *N. gonorrhoeae*, then they may be used as targets for therapeutics to hinder infection.

This project is designed to produce data that will give insight into complement evasion mechanisms used by *N. gonorrhoeae* to persist in the human host. The aim of this study is to test the importance of mCIs on the survival of *N. gonorrhoeae.* Altering the gene expression of the mCIs by using specific miRNAs could help support the hypothesis that mCIs play a role in survivability. Once this data is collected and our results are interpreted, other experiments that cascade from the data we collected will be planned. One of the future aims will be to insert the microRNAs into cells that do not express CD46 or CD55. For this approach, the cell lines will be mutated by deleting the genes of CD46 and CD55. These types of cells are called mCI knockout cell lines. This will provide evidence that the microRNAs specifically target mCIs. If the survival of *N. gonorrhoeae* does not change, then the data will support that the expression of mCIs and survival of *N. gonorrhoeae* are correlated.

Materials and Methods

Cell Lines

MCF7 cells are a model adherent human epithelial cell line. They were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, and 1% L-glutamine with Penicillin-Streptomycin antibiotics. MCF7 cells containing miR-200b and miR200c expression plasmids (miR-200b MCF7 and miR-200c MCF7 respectively) were maintained in DMEM containing 10% fetal bovine serum (FBS), 1% L-glutamine with Penicillin-Streptomycin antibiotics, and 0.5 ug/mL of puromycin. Puromycin was used to select for cells expressing elevated miR-200b and miR-200c. Cell culture media was pre-warmed to 37 °C in the water bath. MCF7 cells were grown in 75 cm^2 cell culture flasks. When the cells were grown for the co-culture serum bacterial assay (ccSBA), they were grown in 12-well dishes. All cell lines were incubated at 37 °C in 5% CO_2 atmosphere. Cells were sub-cultured if they were 90% confluent in the 75 cm^2 cell culture flasks. Since the cells were adherent to the flask, cell density was determined by visualizing the amount of empty space that was present in the flask. Cells were sub-cultured approximately every 5 days. The cell culture media was replaced every two days for feedings. All cell culture was performed in a laminar flow hood.

ME180 cells are a model adherent human endocervical cell line. They were maintained in McCoy's 5A Medium containing 10% fetal bovine serum. ME180 cells containing miR-200b and miR-200c plasmids were maintained in McCoy's 5A Medium containing 10% FBS and 0.5 ug/mL of puromycin. ME180 cells transfected with miR-200b and miR-200c were selected using puromycin. No ccSBA was performed on these

cells due to time limitations. Cell culture media was pre-warmed to 37 °C in the water bath. ME180 cells were grown in 75 cm^2 cell culture flasks. All cell lines were incubated at 37 °C in a 5% CO_2 atmosphere. Cells were sub-cultured if they were 90% confluent in the 75 cm^2 cell culture flasks. All cell culture was performed in a laminar flow hood.

Plasmid

MiR-200c was synthesized from pLenti 4.1 Ex miR200c-141 which was created by Greg Goodall (Addgene plasmid # 35534 ; http://n2t.net/addgene:35534 ; RRID:Addgene_35534)[63]. MiR-200b was synthesized from pLenti 4.1 Ex miR200b-200a-429 which was created by Greg Goodall (Addgene plasmid # 35533 ; http://n2t.net/addgene:35533 ; RRID:Addgene_35533)[63]. MiR-200b was amplified and inserted back into the plasmid backbone after excising miR-200a and miR-429 to create the miR-200b MCF7 cell line. Monarch PCR & DNA Cleanup Kit were used to obtain the plasmid after a PCR. Restriction enzymes EcoR1 and Xho1 were used. MiR-200c was amplified and inserted back into the plasmid backbone after excising miR-141 to create the miR-200c MCF7 cell line. Restriction enzymes EcoR1 and Not1 were used. The digested plasmid and insert were then run on a gel. The E.Z.N.A Gel Extraction Kit by Omega was used to extract the plasmid and insert from the gel before ligation. The product of the ligation was transformed into OneShot Top10 Competent *Escherichia coli* (*E. coli*).

Transient Transfection

Plasmid DNA was transfected into cells using FuGENE HD transfection reagent according to the instructions of the manufacturer. Cells were plated in a 10-cm dish and incubated overnight. Puromycin was used for selection for MCF7 and ME180 cells expressing altered miR-200b or miR-200c levels. The selection process took approximately two weeks. After cells were selected for, they were transferred into a 175 cm^2 cell culture flasks where they were expanded under selection until optimal density. The cells were then collected and frozen back. These cells were stored in liquid nitrogen until further use.

Bacterial Strain

MS11 is a *N. gonorrhoeae* strain isolated from The Mount Sinai Hospital in New York City, NY. The MS11 strain is a wildtype strain used in Dr. Weyand's lab. MS11 was streaked out on GCB agar plates which were made two days in advance. Plates were incubated overnight at 37 °C in a 5% CO_2 atmosphere incubator. After 24 hours, five colonies from the overnight plate were then lawned on another GCB agar plate. For the ccSBA, the lawn was then used to lawn on another GCB plate 6 hours prior to the experiment.

Total RNA Isolation and Preparation for RT-qPCR

Non-transfected wildtype MCF7 cells will now be referred to as MCF7 WT. Wildtype MCF7 cells transfected with the plasmid containing miR-200c will now be referred to as miR-200c MCF7. Wildtype MCF7 cells transfected with the plasmid

containing miR-200b will now be referred to as miR-200b MCF7. All three cell lines were grown in 75 cm^2 cell culture flasks containing media (previously described) for RNA isolation. Once the cells reached 90% confluency, they were collected in a 15 mL conical tube. RNA isolation was performed using the RNA Isolation Kit from Invitrogen according to the manufacturer's recommended protocol. After the RNA was isolated, an RNA Qubit Assay was performed using the Invitrogen Qubit Fluorometer to obtain the concentration of the collected RNA.

After obtaining the concentration of RNA collected from each cell line, a DNase treatment was performed. The DNA-free Kit: DNase Treatment and Removal by Invitrogen was used. This was performed to eliminate any DNA contamination within the RNA samples. After the protocol was completed, another RNA Qubit Assay was performed using the Invitrogen Qubit Fluorometer to obtain the concentration of the collected RNA. The resulting concentration was slightly decreased relative to the initial concentration following this purification step. Samples were then transferred to the Ohio University Genomics Facility for a Bioanalyzer experiment. RNA Integrity Number (RIN) was obtained from the Bioanalyzer. Only samples scoring a RIN value above 7 were used. RIN values range from 0-10.

Complementary DNA (cDNA) synthesis was performed on RNA samples with high RIN values. iScript cDNA Synthesis Kit by Bio-Rad was used to perform the cDNA synthesis. RT-qPCR samples were prepared according to manufacturer's recommended protocol in 96-well dishes using PrimeTime Gene Master Mix and PrimeTime Assay Primers from ID Technology. Housekeeping genes GAPDH and YWHAZ were used to normalize the results. A negative control was prepared using water as a no-template

sample. Plates were then submitted to the Ohio University Genomics Facility to perform the RT-qPCR experiment. Statistical analysis was done by the Student's T-Test on Prism.

Flow Cytometry

Cells were grown in 75 cm^2 cell culture flasks until they were 85-95% confluent. The protocol for cell preparation for flow cytometry was based on the current protocol by Maciorowski et al[64]. Cells from the monolayer were collected at a concentration of 1 million cells/mL into a conical tube using TrypLe by Gibco and neutralized with complete media. The cells were then centrifuged until they formed a pellet at the bottom of the conical tube. Subsequently, the cells were washed with ice-cold phosphate-buffer saline (PBS). The Weyand Lab uses purchased commercial antibodies conjugated to different fluorophores targeting the mCIs. Fluorescein isothiocyanate (FITC), allophycocyanin (APC), and phycoerythrin (PE) conjugated to specific mCIs were obtained from BioLegend and were used as fluorophores to detect mCI protein expression. BioLegend manufactured FITC conjugated to mouse-anti-CD46 monoclonal antibody clone MEM-258 to detect surface expression of CD46. APC was conjugated to mouse-anti-CD55 monoclonal antibody clone JS11 to observe surface expression of CD55. PE was conjugated to mouse-anti-CD59 monoclonal antibody close p282 (H19) to detect surface expression of CD59. The cells were incubated on ice for 30 minutes with PBS containing 2.5 μg/mL of either FITC, APC, or PE for staining. A portion of cells were also stained with 1 μL/mL eFluor™ 780 fixable viability dye by eBioscience™. This dye was used to observe if cells were viable.

Compensation is a necessary control which minimizes the rate of false positives due to cross-detection of fluorophores in multiple channels. First, a single stain compensation for FITC, PE, and APC using UltraComp eBeads from Thermofischer was performed according to the manufacturer's recommended protocol. Then, a heterogenous population of live and dead cells was prepared to standardize detection of eFluor 780 dye according to the manufacturer's recommendation. Finally, fluorescence minus one (FMO) controls were prepared, which omit individual fluorophores to determine the antibody cocktail's effect on the detection channel of single fluorophores. All three steps are done to help determine that the detection of signal is due to the binding of the target primary antibody and not a false positive. During the 30-minute incubation, the cells were protected from light. Unstained cells were also prepared to compensate for natural fluorescence of cells. After the incubation, cells were washed one time with PBS and then resuspended in 0.2mL of PBS for flow cytometry. Compensation was calculated using AutoSpill algorithm and applied to the analyzed samples in FlowJo software by Becton, Dickinson, and Company. Calculations and analysis were performed to obtain the normalized mean fluorescence intensity (MFI) for each sample. Statistical analysis was done by the Student's T-Test on Prism.

Co-culture Serum Bacterial Assay (ccSBA)

The co-culture serum bacterial assay was the method used to test the survivability of *N. gonorrhoeae* when different cells were infected. *N. gonorrhoeae* was struck out two days before the experiment on GCB plates. Five colonies were chosen to lawn 24 hours after the streak. Six hours before the infection, another lawn was performed using the first

lawn. All plates were incubated at 37°C in a 5% CO_2 atmosphere incubator. Cells were grown in 12-well dishes until optimal density.

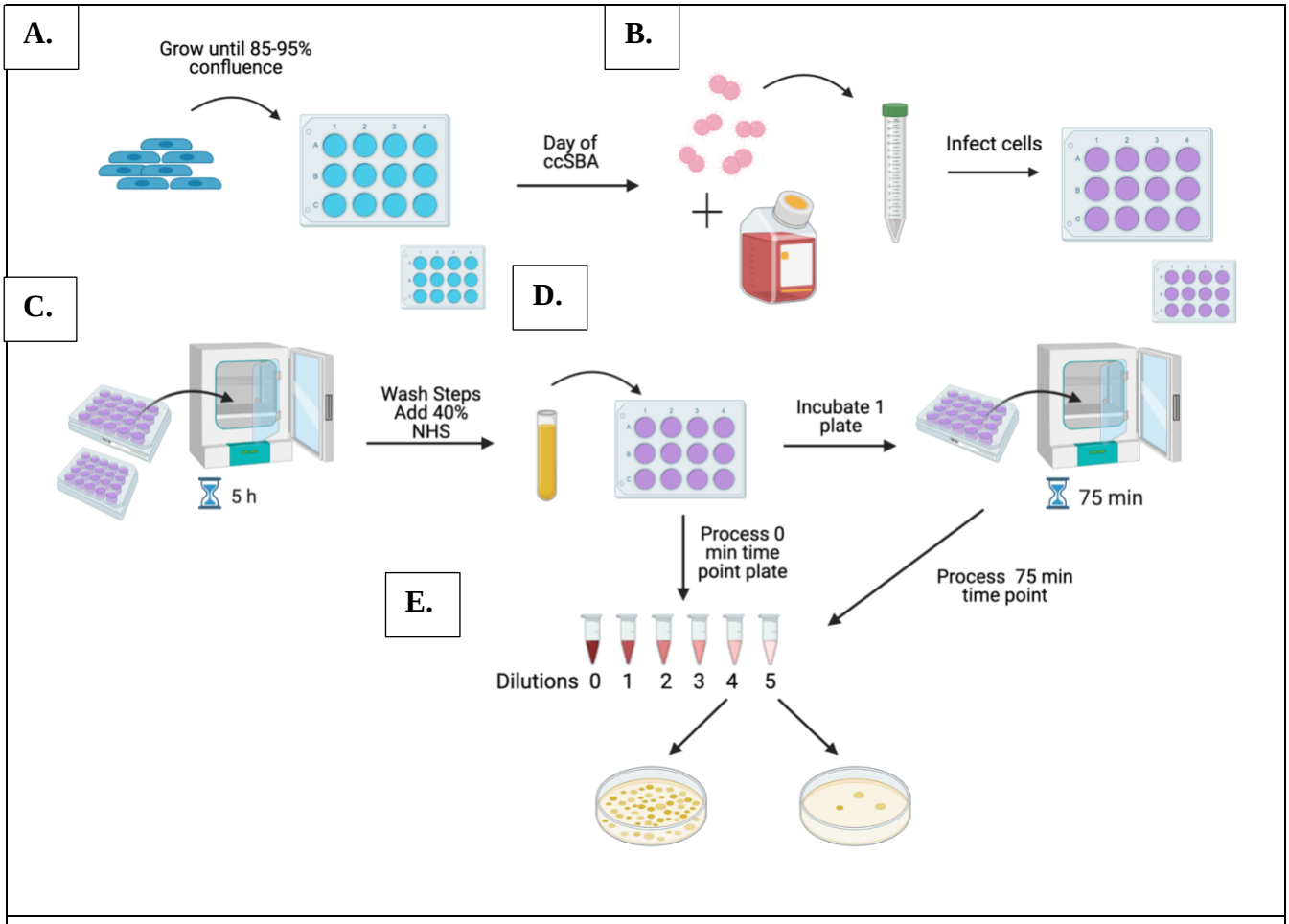


Figure 8. Visual representation of the co-culture serum bacterial assay (ccSBA). Panel A illustrates growth of the MCF7 cell lines in two 12-well plates. Panel B depicts preparation of *N. gonorrhoeae* inoculum and infection of cells. Panel C shows the infected 12-well plates being placed into an incubator for 5 hours. Panel D shows addition of NHS into 12-well plates. The 12-well plate that is placed back into the incubator is the 75-min time point plate. The NHS is aspirated off the 0-min time point plate immediately and begins processing. Panel E illustrates the dilution series obtained from the 12-well plate. Dilutions 4 and 5 were plated to grow the *N. gonorrhoeae* microcolonies in countable range (30-300). Image not to scale. Created by Biorender.com

The three cell lines, MCF7 WT, miR-200c MCF7, and miR-200b MCF7 were all grown in duplicate in two 12-well plates. After collecting the 6-hour lawn, the optic density (OD) was measured. Calculations were performed to normalize the number of bacteria used for infection assays. The monolayers of the human epithelial cells were infected with *N. gonorrhoeae* and grown in co-culture. After infecting duplicate 12-well plates, the plates were placed into the incubator for five hours. After the five-hour timepoint, polarized light images of the wells were taken using the Leica DMi1 microscope. When the five-hour time point was over, the two plate were removed from the incubator. The infected wells were washed with 1mL of serum-free media (DMEM) to remove nonadherent bacteria. In the 75-minute time point plate, 500mL of 40% normal human serum (NHS) was added to challenge the infected cells. This plate was placed back into the incubator for another 75 minutes. The NHS was collected from different donors and possessed complement system proteins like the MAC complex. For consistency, NHS was added to the 0-minute time point plate and immediately removed. The infected wells on the 0-min time point plate were washed with 1mL of GCB. Vacuum-filtered saponin in GCB was used to unadhere the cells from the plate. 500 μL of saponin in GCB was added into each well and placed into the incubator for another 10 minutes. After 10 minutes, wells were scraped in different directions to transfer the cells in saponin and GCB into a microfuge tube containing 500 μL of GCB. These microfuge tubes are the 0 dilution. In Figure 8, the following steps are referred to as processing plates. Each microfuge tube containing cells from one well was vortexed for a minute. 100 μL of the 0 dilution was transferred into another microfuge tube containing 900 μL of GCB. These microfuge tubes are the first dilution. The tubes were vortexed for 1 minute

and 100 μL of the first dilution was transferred into another microfuge tube containing 900 μL of GCB. These tubes were the second dilution. This process repeated until five dilutions were made. After all 5 dilutions were made, only the fourth and fifth dilutions were plated onto GCB plates. For the fourth dilution, 50 μL were plated. For the fifth dilution, 100 μL were plated. After infecting the GCB plates with the diluted bacteria, the GCB plates were placed into the incubator for 48 hours. Two dilutions were plated to increase the probability of obtaining colony forming units in the countable range between 30-300. After 75 minutes of incubating the 75-minute time point plate, the same procedure which was performed on the 0-minute time point plate was performed on the 75-minute time point plate.

The colony forming units were determined at the addition of serum and after 75 minutes of treatment with serum. After 48 hours of incubation, the number of colonies that had formed on each plate were then counted. A statistical analysis was performed on the data to detect the difference in colony growth between the three cell lines. The comparison of the 0-minute time point and the 75-minute time point was used to monitor the survival of *N. gonorrhoeae* in the presence of human serum. Figure 8 shows a summary of the ccSBA procedure. Statistical analysis was done by the Student's T-Test on Prism.

A.

Sequence ID: **Query_52989** Length: **1799** Number of Matches: **1**

Range 1: 1429 to 1523 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
171 bits(92)	5e-47	94/95(99%)	0/95(0%)	Plus/Plus

```
Query  1     CCAGCTCGGGCAGCCGTGGCCATCTTACTGGGCAGCATTGGATGGAGTCAGGTCTCTAAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1429  CCAGCTCGGGCAGCCGTGGCCATCTTACTGGGCAGCATTGGATGGAGTCGGGTCTCTAAT  1488

Query  61    ACTGCCTGGTAATGATGACGGCGGAGCCCTGCACG  95
             |||||||||||||||||||||||||||||||||||
Sbjct  1489  ACTGCCTGGTAATGATGACGGCGGAGCCCTGCACG  1523
```

B.

Sequence ID: **Query_61263** Length: **1977** Number of Matches: **1**

Range 1: 831 to 898 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
126 bits(68)	8e-34	68/68(100%)	0/68(0%)	Plus/Plus

```
Query  1     CCCTCGTCTTACCCAGCAGTGTTTGGGTGCGGTTGGGAGTCTCTAATACTGCCGGGTAAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  831   CCCTCGTCTTACCCAGCAGTGTTTGGGTGCGGTTGGGAGTCTCTAATACTGCCGGGTAAT  890

Query  61    GATGGAGG  68
             ||||||||
Sbjct  891   GATGGAGG  898
```

Figure 9. Panel A shows the NCBI alignment for miR-200b (query) and the consensus sequence output (subject) using Sanger sequencing on the plasmid containing the microRNA. Panel B exhibits the NCBI alignment for miR-200c (query) and the consensus sequence output (subject) using Sanger sequencing[65].

The plasmids containing the microRNAs that were acquired from Addgene had to be modified. Each original plasmid contained at least one microRNA that had to be excised. This was done to create plasmids that only expressed miR-200b or miR-200c. After the plasmids containing only the microRNA were cloned, they were sequenced. Multiple primers were used to generate a forward or reverse strand. A consensus sequence of the plasmid was acquired by overlapping the output of the forward and reverse primers. The sequence in Figure 9 was generated from one forward primer and one reverse primer. The query in each panel is the sequence for the microRNA. The

subject is the output of the sequence generated from two different primers. The sequence for miR-200b has one base pair that does not align.

After confirming that the target microRNA sequence was present in the modified plasmid, we conducted a real-time quantitative polymerase chain reaction (RT-qPCR). The housekeeping gene YWHAZ was used to normalize the data[66]. As stated in the materials and methods section, the miR-200c MCF7 cell line was transfected with the plasmid containing miR-200c and the miR-200b MCF7 cell line was transfected with the plasmid containing miR-200b. Figure 10A shows that there was a significant difference in the expression of miR-200b between MCF7 WT and miR-200b MCF7. The figure also shows that there was a significant difference in the expression of miR-200c between MCF7 WT and miR-200c MCF7 in Figure 10B. Specifically, Figure 10B also shows that there is significantly less miR-200c in miR-200b MCF7 than there is in the MCF7 WT as well. The transcript levels for the mCIs in all three cell lines had no significant difference.

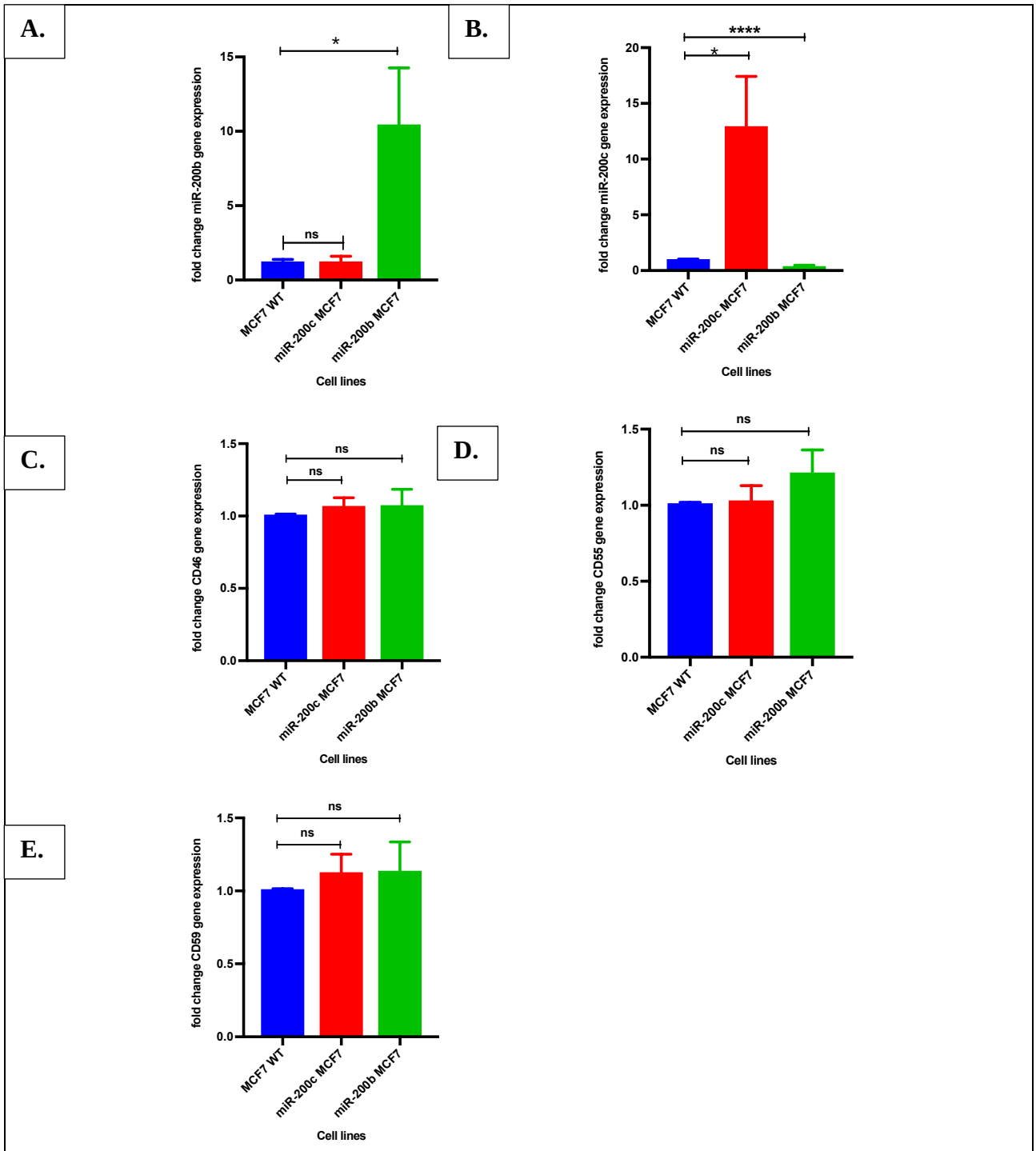


Figure 10. Results for RT-qPCR on miR-200b, miR-200c, CD46, CD55, and CD59. miR-200c MCF7 and miR-200b MCF7 were compared to MCF7 WT ($n = 5$, $p \leq 0.05$). Housekeeping gene YWHAZ was used to normalize the data. Student's T-Test was used for statistical analysis.

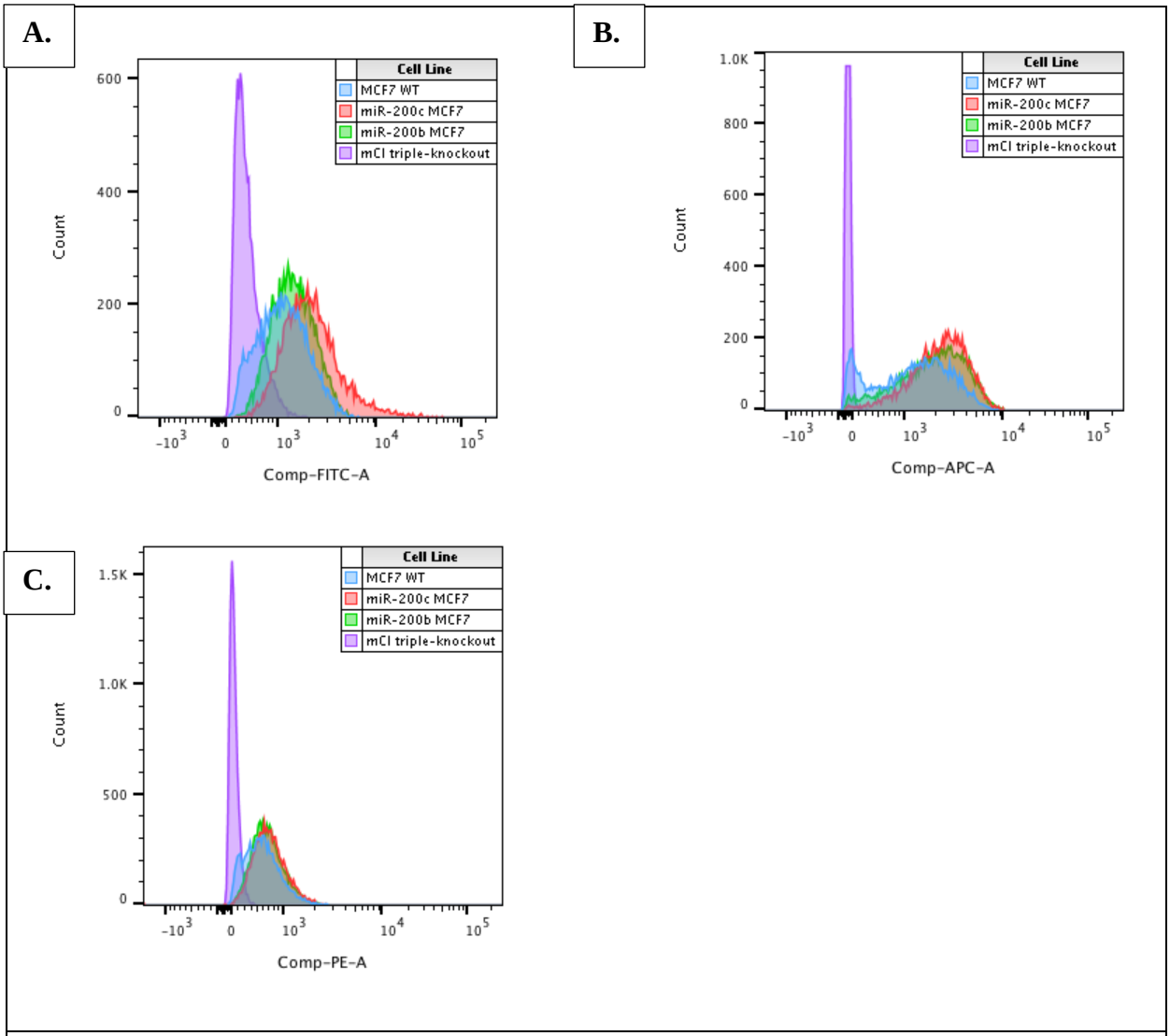


Figure 11. Flow cytometry histograms demonstrating surface expression of CD46, CD55, and CD59 in all cell lines. Fluorescein isothiocyanate (FITC) was used to detect surface expression of CD46. Allophycocyanin (APC) was used to detect surface expression of CD55. Phycoerythrin (PE) was used to detect surface expression of CD59. mCI triple-knockout cells were used as a negative control.

Flow cytometry was performed on the three cell lines to detect if there was a difference in surface expression of CD46, CD55, and CD59. All cells were tested for viability using eFluor™ 780 fixable viability dye. All cells were viable (data not shown).

FITC, APC, and PE were conjugated to different primary antibodies that bound to specific mCIs. FITC stained for CD46, APC stained for CD55, and PE stained for CD59. Figure 11 shows the number of cells that were fluorescent for each stain. mCI triple-knockout cells are modified MCF7 cells. The mCI triple-knockout cells do not have any of the mCIs present in their genome. Figure 12 quantifies the results seen in the histograms of Figure 11. The fluorescence of each cell line was compensated with unstained cells. The normalized mean fluorescence intensity was used to quantify the surface expression of each mCI. Figure 12A shows that neither miR-200b MCF7 nor miR-200c MCF7 had any significant surface expression of CD46 compared to MCF7 WT. mCI triple-knockout, which lack mCIs, had significantly lower expression of CD46 than the MCF7 WT. This result is similar in all of the mCIs shown in Figure 12.

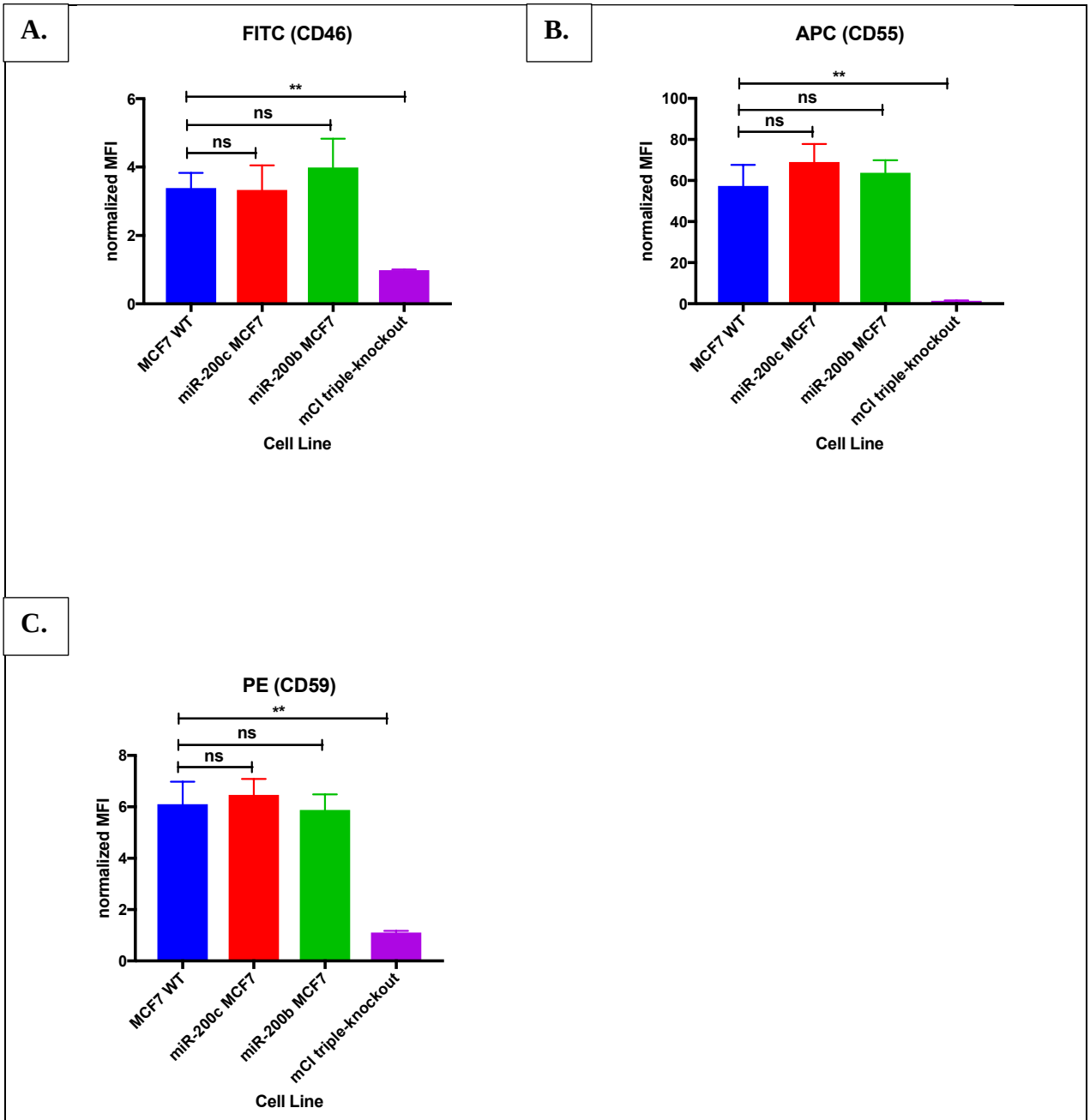


Figure 12. Quantification of expression of CD46, CD55, and CD59 within each cell line. Expression was compared to MCF7 WT ($n = 3$, $p \leq 0.05$). mCI triple-knockout cells served as a negative control. Normalized MFI is the mean fluorescence intensity of each fluorophore after normalizing the baseline fluorescence of the fluorophore. Student's T-Test was used for statistical analysis.

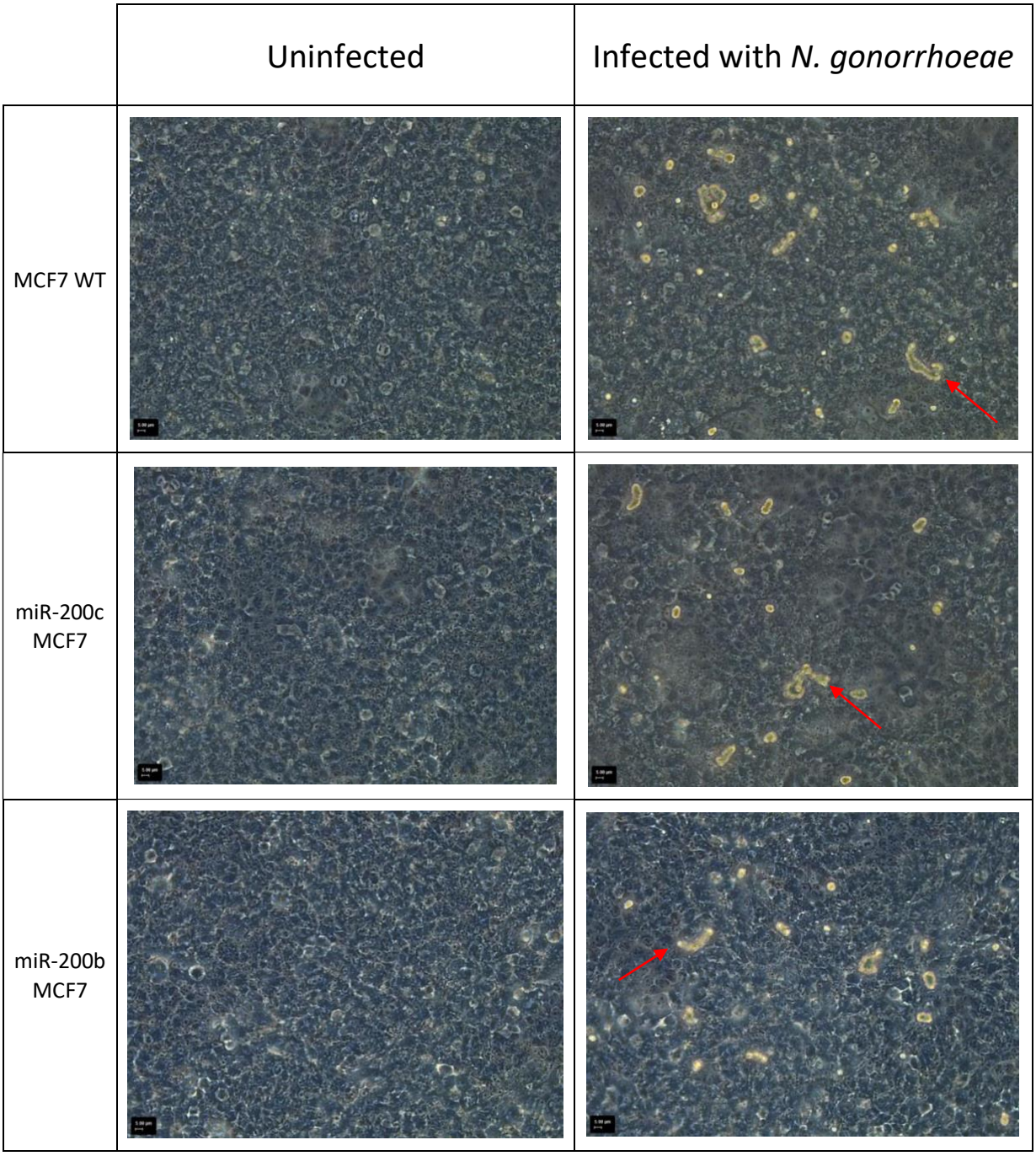


Figure 13. Polarized light images of MCF7 WT, miR-200b MCF7, and miR-200c MCF7 uninfected and after 5 hours of *N. gonorrhoeae* infection. The red arrows are pointing to microcolonies of *N. gonorrhoeae* attached to the human cells. The images were taken at 20X magnification.

Unexpectedly, after characterizing the cell lines, we found no change in mCI expression within the cells that contained the plasmids with microRNAs. These microRNAs have been predicted to upregulate the expression of CD46 and CD55, but not CD59. However, we proceeded to test if the survivability of *N. gonorrhoeae* had any change due to the altered expression of the microRNAs. Figure 13 complies the pictures taken of each cell line infected and uninfected with wild-type *N. gonorrhoeae.* The yellow clusters seen in the infected pictures are microcolonies of *N. gonorrhoeae* after 5 hours of infection. Figure 14 shows the percent survivability of *N. gonorrhoeae* after exposure to complement in the ccSBA.

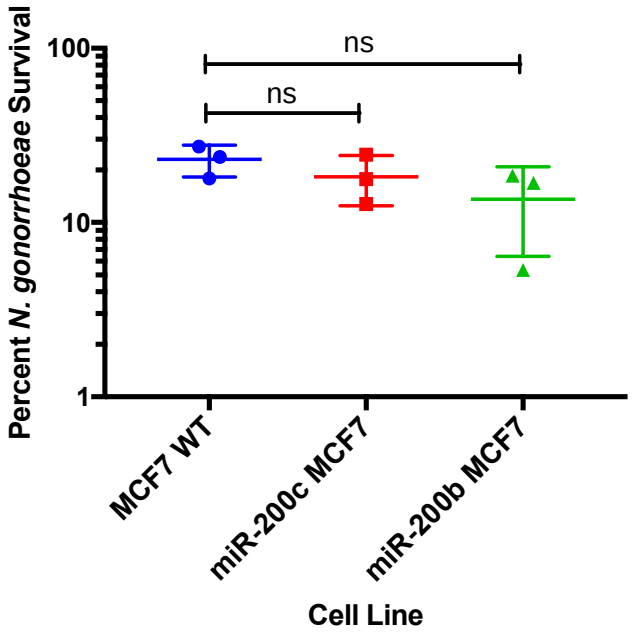


Figure 14. Percent survivability of *N. gonorrhoeae* in MCF7 WT, miR-200c MCF7, and miR-200b MCF7 after using the co-culture serum bacterial assay protocol. Survivability of miR-200b MCF7 and miR-200c MCF7 were compared to MCF7 WT ($n = 3$, $p \leq 0.05$). Student's T-test was used for statistical analysis.

The results of the ccSBA showed that there was no significant difference between miR-200c MCF7 and MCF7 WT. The results also show that miR-200b MCF7 and MCF7 WT do not have a significant difference as well. Therefore, miR-200b does not increase the expression of CD46 or CD55, which in turn, results in no change of survivability for *N. gonorrhoeae.* A similar statement can be made for miR-200c. This data does not support the hypothesis made based on the data presented by Hillman et al[1].

Discussion

Overall, the results of this study showed that overexpression of miR-200b and miR-200c individually do not increase or decrease the protein expression levels of mCIs CD46, CD55, and CD59. Since the mCI expression did not change, we were still not able to correlate expression of the mCIs with changes in *N. gonorrhoeae* survival.

The results shown in Figure 9 are the alignment sequences for the microRNA and the sequence output using Sanger sequencing method. For miR-200b, there was one base pair that was mismatched. Although one base pair can change the function of a protein, microRNAs do not undergo translation. They work by binding to their complementary target. One base change may have not affected the function of the microRNA, but further testing should be done. Correcting the gene sequence may be beneficial in a future study.

Figure 10 displays the results of the RT-qPCR. Figure 10A shows that miR-200b MCF7 cells have significantly more transcripts of miR-200b than the MCF7 WT cells. Shown in Figure 10B, miR-200c MCF7 cells have increased transcripts of miR-200c compared to MCF7 WT cells. This data showed that the plasmid transfection had been successful. The transcript levels for CD46, CD55, and CD59 had no significant

difference in the cells overexpressing microRNAs compared to the MCF7 WT cells. This meant that there was no increase in mCI transcript levels between any of the cell lines. Therefore, the microRNAs were not affecting the levels of mCI transcription.

Flow cytometry was conducted on all of the cell lines to observe if there was an effect on human cell surface expression of mCIs. Figure 11 shows the results of flow cytometry in histogram format. This data is not quantitative but is used to visually represent the change in fluorescence of different fluorophores. In Figure 11A, cell lines MCF7 WT, miR-200b MCF7, and miR-200c MCF7 had overlapping fluorescence in the histograms. Fluorescence for mCI triple-knockout cells were shifted to the left. Similar trends were seen in Figure 11B and 11C as well. The mean fluorescence intensity (MFI) was calculated for FITC, APC, and PE. FITC, APC, and PE are fluorophores that were conjugated to specific mCI antibodies commercially. FITC is a dye that was used to detect surface expression of CD46 in this study. APC is a dye that was used to detect surface expression of CD55. PE is a dye that was used to detect surface expression of CD59. The quantitative results from the flow cytometry experiment can be seen in Figure 12. The normalized MFI can be used as a way to observe cell surface expression of the different mCIs. For example, if the normalized MFI values are low, there is less fluoresce detected for the particular protein. Hence, the surface expression levels of a particular protein must also be low. The normalized MFI values are correlated to the mCI surface expression. The results in Figure 12 show that there is no significant difference in cell surface expression of CD46, CD55, and CD59 in the cells overexpressing microRNAs when compared to MCF7 WT. mCI triple-knockout cells had significantly less surface expression of all three mCIs. This cell line was used as a negative control because the

mCI triple-knockout cells do not express mCIs. This data showed that the cells overexpressing microRNAs did not have higher expression of mCIs, contrary to the hypothesis. Therefore, the results of this experiment did not correlate with the results obtained by Hillman et al[1]. Their study showed an increase in protein synthesis of CD46 and CD55. A possible reason for not observing an increase in mCI expression could be due to the differences of cell lines used in the study conducted by Hillman et al and MCF7 cells[1]. Another possible reason for no significant increase in expression could be because upregulation by microRNAs occurs in specific environments, like quiescence[46]. In our study, cell proliferation was occurring during all experimentation.

After the RT-qPCR and flow cytometry experiments showed no significant difference in mCIs, we wanted to test if there was increased survivability of *N. gonorrhoeae* between the different cell lines. Although the microRNAs seemed to not affect mCI expression, they may have been affecting other proteins that could have altered survivability of *N. gonorrhoeae* during the ccSBA. Figure 13 shows polarized images of the three cell lines captured after 5 hours of infection with *N. gonorrhoeae.* These were taken to ensure that the bacteria were forming microcolonies on the human cells. No qualitative difference in microcolony formation was detected. The data exhibited in Figure 14 illustrates that there is no significant difference in survival of *N. gonorrhoeae* between the cells overexpressing microRNAs and MCF7 WT cells.

Altogether, the data in this study shows that overexpressing a single microRNA that was predicted to increase expression of multiple mCIs had no effect in MCF7 cells. Consequently, there was no observed difference in survival of *N. gonorrhoeae* when challenged with normal human serum. This result could be due to MCF7 cells needing

multiple different microRNAs to result in significant increased expression of mCIs. For example, inserting a plasmid containing miR-200b, miR-200c, and miR-217 into MCF7 WT cells may collectively result in an increase in mCI expression. Other microRNAs, shown in Table 2, can also be tested for their effect on mCI expression. As mentioned previously, Target Scan and DIANA Tools predict the targets of microRNAs[47,48]. Using these databases may also help find microRNAs which increase mCI expression.

Multiple methods have been used to increase protein expression in mammalian cells. One method is by ensuring the target protein has a strong promotor because it will produce large amounts of the target protein[67]. In mammalian cells, CMV protomers are typically used. Plasmids containing the microRNAs had CMV promoters, but we have not studied promoters used by mCIs in the MCF7 cells. Another method that can be used to increase protein expression is by inhibiting different silencing or downregulating processes[67]. These processes may include microRNAs that the cell is using to downregulate expression of mCIs. By inhibiting or interfering with these mechanisms, increased expression may occur. Inserting a plasmid containing the target protein with a strong promoter is also another method that can be used to increase expression of different proteins.

Future Directions

Future efforts will need to be made to identify an approach that increases mCI expression. The results of this thesis encourage the exploration of different microRNAs that are predicted to influence mCI expression. The current results suggest that upregulation of one microRNA may not be sufficient to alter gene expression of CD46,

CD55, and CD59 in MCF7 cells. A continuation of this study can explore the overexpression of multiple microRNAs predicted to target one protein. For example, overexpressing both miR-200b and miR-200c in MCF7 cells. This could also be done by transfecting the cells with the original plasmid obtained from Addgene. Another possible direction from this study is to overexpress miR-200b and miR-200c in additional cell lines such as ME180 cells. Currently, the Weyand Lab is generating ME180 cells overexpressing miR-200b and miR-200c using a similar approach as used in this study. This would allow for a more general conclusion on the effect these specific microRNAs on mCI expression. It may also be beneficial to conduct a similar study in the same cell lines as used by Hillman et al[1]. This would help characterize the function of the modified Addgene plasmids. The Weyand Lab is also pursuing another upregulation approach by overexpressing Hepatitis B X-interaction protein (HBXIP). HBXIP has shown to upregulate mCIs in MCF7 cells[55]. All of these methods may be used in the future to further investigate the role of mCIs in *N. gonorrhoeae* survival.

References

1. Hillman, Y., Mazkereth, N., Farberov, L., Shomron, N. & Fishelson, Z. Regulation of Complement-Dependent Cytotoxicity by MicroRNAs miR-200b, miR-200c, and miR-217. *J. Immunol.* **196**, 5156–5165 (2016).
2. Unemo, M. *et al.* Gonorrhoea. *Nat. Rev. Dis. Primer* **5**, 79 (2019).
3. U.S. Centers for Disease Control and Prevention. Gonorrhea - 2018 Sexually Transmitted Diseases Surveillance. https://www.cdc.gov/std/stats18/gonorrhea.htm (2019).
4. U.S. Centers for Disease Control and Prevention. About Antibiotic Resistance. https://www.cdc.gov/drugresistance/about.html (2020).
5. U.S. Centers for Disease Control and Prevention. Antibiotic Resistance Threats in the United States 2019. https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf (2019).
6. Lefebvre, B. *et al.* Ceftriaxone-Resistant *Neisseria gonorrhoeae* , Canada, 2017. *Emerg. Infect. Dis.* **24**, 381–383 (2018).
7. Mayo Clinic. Gonorrhea. https://www.mayoclinic.org/diseases-conditions/gonorrhea/diagnosis-treatment/drc-20351780 (2019).
8. Unemo, M. & Shafer, W. M. Antimicrobial resistance in Neisseria gonorrhoeae in the 21st century: past, evolution, and future. *Clin. Microbiol. Rev.* **27**, 587–613 (2014).
9. World Health Organization. Antibiotic-resistant gonorrhoea on the rise, new drugs needed. https://www.who.int/news-room/detail/07-07-2017-antibiotic-resistant-gonorrhoea-on-the-rise-new-drugs-needed (2017).

10. Pérez Medina, K. & Dillard, J. Antibiotic Targets in Gonococcal Cell Wall Metabolism. *Antibiotics* **7**, 64 (2018).

11. John Tapsall. World Health Organization: Antimicrobial resistance in Neisseria gonorrhoeae. 65 (2001).

12. Unemo, M. & Shafer, W. M. Antibiotic resistance in Neisseria gonorrhoeae: origin, evolution, and lessons learned for the future. *Ann. N. Y. Acad. Sci.* **1230**, E19-28 (2011).

13. Ilina, E. N. *et al.* Mutation in ribosomal protein S5 leads to spectinomycin resistance in Neisseria gonorrhoeae. *Front. Microbiol.* **4**, (2013).

14. Donà, V. *et al.* Multiplex Real-Time PCR Assay with High-Resolution Melting Analysis for Characterization of Antimicrobial Resistance in Neisseria gonorrhoeae. *J. Clin. Microbiol.* **54**, 2074–2081 (2016).

15. Anderson, M. T. & Seifert, H. S. Opportunity and Means: Horizontal Gene Transfer from the Human Host to a Bacterial Pathogen. *mBio* **2**, (2011).

16. Kim, W. J. *et al.* Neisseria gonorrhoeae evades autophagic killing by downregulating CD46-cyt1 and remodeling lysosomes. *PLOS Pathog.* **15**, e1007495 (2019).

17. Edwards, J. L. The role of complement in gonococcal infection of cervical epithelia. *Vaccine* **26**, I56–I61 (2008).

18. Kim, D. D. & Song, W.-C. Membrane complement regulatory proteins. *Clin. Immunol.* **118**, 127–136 (2006).

19. Riley-Vargas, R. C., Gill, D. B., Kemper, C., Liszewski, M. K. & Atkinson, J. P. CD46: expanding beyond complement regulation. *Trends Immunol.* **25**, 496–503 (2004).

20. Lublin, D. M. & Atkinson, J. P. Decay-Accelerating Factor: Biochemistry, Molecular Biology, and Function. *Annu. Rev. Immunol.* **7**, 35–58 (1989).

21. Huang, Y., Qiao, F., Abagyan, R., Hazard, S. & Tomlinson, S. Defining the CD59-C9 Binding Interaction *. *J. Biol. Chem.* **281**, 27398–27404 (2006).

22. Weyand, N. J. *et al.* Monoclonal Antibody Detection of CD46 Clustering beneath Neisseria gonorrhoeae Microcolonies. *Infect. Immun.* **74**, 2428–2435 (2006).

23. Gill, D. B., Spitzer, D., Koomey, M., Heuser, J. E. & Atkinson, J. P. Release of host-derived membrane vesicles following pilus-mediated adhesion of Neisseria gonorrhoeae: Neisseria gonorrhoeae-induced host cell vesiculation. *Cell. Microbiol.* **7**, 1672–1683 (2005).

24. Källström, H. *et al.* Attachment of Neisseria gonorrhoeae to the cellular pilus receptor CD46: identification of domains important for bacterial adherence. *Cell. Microbiol.* **3**, 133–143 (2001).

25. Kirchner, M., Heuer, D. & Meyer, T. F. CD46-Independent Binding of Neisserial Type IV Pili and the Major Pilus Adhesin, PilC, to Human Epithelial Cells. *Infect. Immun.* **73**, 3072–3082 (2005).

26. Tagliabue, G. The central dogma, "GMO" and defective epistemology. *GM Crops Food* **8**, 209–215 (2017).

27. Cannell, I. G., Kong, Y. W. & Bushell, M. How do microRNAs regulate gene expression? *Biochem. Soc. Trans.* **36**, 1224–1231 (2008).

28. McCall, M. N. *et al.* Toward the human cellular microRNAome. *Genome Res.* **27**, 1769–1781 (2017).

29. Plotnikova, O., Baranova, A. & Skoblov, M. Comprehensive Analysis of Human microRNA–mRNA Interactome. *Front. Genet.* **10**, (2019).

30. Peng, Y. & Croce, C. M. The role of MicroRNAs in human cancer. *Signal Transduct. Target. Ther.* **1**, 15004 (2016).

31. LeTonqueze, O., Lee, J. & Vasudevan, S. MicroRNA-mediated posttranscriptional mechanisms of gene expression in proliferating and quiescent cancer cells. *RNA Biol.* **9**, 871–880 (2012).

32. Ameres, S. L. & Zamore, P. D. Diversifying microRNA sequence and function. *Nat. Rev. Mol. Cell Biol.* **14**, 475–488 (2013).

33. Valinezhad Orang, A., Safaralizadeh, R. & Kazemzadeh-Bavili, M. Mechanisms of miRNA-Mediated Gene Regulation from Common Downregulation to mRNA-Specific Upregulation. *Int. J. Genomics* **2014**, 1–15 (2014).

34. Truesdell, S. S. *et al.* MicroRNA-mediated mRNA Translation Activation in Quiescent Cells and Oocytes Involves Recruitment of a Nuclear microRNP. *Sci. Rep.* **2**, 842 (2012).

35. Vasudevan, S., Tong, Y. & Steitz, J. A. Cell cycle control of microRNA-mediated translation regulation. *Cell Cycle* **7**, 1545–1549 (2008).

36. Vasudevan, S., Tong, Y. & Steitz, J. A. Switching from Repression to Activation: MicroRNAs Can Up-Regulate Translation. *Science* **318**, 1931–1934 (2007).

37. Ørom, U. A., Nielsen, F. C. & Lund, A. H. MicroRNA-10a Binds the 5′UTR of Ribosomal Protein mRNAs and Enhances Their Translation. *Mol. Cell* **30**, 460–471 (2008).

38. Lin, C.-C. *et al.* A KLF4-miRNA-206 Autoregulatory Feedback Loop Can Promote or Inhibit Protein Translation Depending upon Cell Context. *Mol. Cell. Biol.* **31**, 2513–2527 (2011).

39. Iwasaki, S. & Tomari, Y. Argonaute-mediated translational repression (and activation). *Fly (Austin)* **3**, 205–208 (2009).

40. Henke, J. I. *et al.* microRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J.* **27**, 3300–3310 (2008).

41. Fehr, C., Conrad, K. D. & Niepmann, M. Differential stimulation of hepatitis C virus RNA translation by microRNA-122 in different cell cycle phases. *Cell Cycle* **11**, 277–285 (2012).

42. Roberts, A. P. E., Lewis, A. P. & Jopling, C. L. miR-122 activates hepatitis C virus translation by a specialized mechanism requiring particular RNA components. *Nucleic Acids Res.* **39**, 7716–7729 (2011).

43. Mohr, A. & Mott, J. Overview of MicroRNA Biology. *Semin. Liver Dis.* **35**, 003–011 (2015).

44. Wu, L. & Belasco, J. G. Let Me Count the Ways: Mechanisms of Gene Regulation by miRNAs and siRNAs. *Mol. Cell* **29**, 1–7 (2008).

45. Vasudevan, S. & Steitz, J. A. AU-Rich-Element-Mediated Upregulation of Translation by FXR1 and Argonaute 2. *Cell* **128**, 1105–1118 (2007).

46. Vasudevan, S. Posttranscriptional Upregulation by MicroRNAs: Posttranscriptional Upregulation by MicroRNAs. *Wiley Interdiscip. Rev. RNA* **3**, 311–330 (2012).

47. Agarwal, V., George W. Bell, Jin-WU Nam, & David P. Bartel. Target Scan Human: Prediction of microRNA targets. http://www.targetscan.org/vert_72/ (2015).

48. Karagkouni, D. *et al.* DIANA-TarBase v8: a decade-long collection of experimentally supported miRNA–gene interactions. *Nucleic Acids Res.* **46**, D239–D245 (2018).

49. Lee, A. V., Oesterreich, S. & Davidson, N. E. MCF-7 Cells--Changing the Course of Breast Cancer Research and Care for 45 Years. *JNCI J. Natl. Cancer Inst.* **107**, djv073–djv073 (2015).

50. Soule, H. D., Vazquez, J., Long, A., Albert, S. & Brennan, M. A Human Cell Line From a Pleural Effusion Derived From a Breast Carcinoma 2. *JNCI J. Natl. Cancer Inst.* **51**, 1409–1416 (1973).

51. MCF7 ATCC® HTB-22™. https://www.atcc.org/products/all/htb-22.aspx#generalinformation.

52. Cass Glodek. A History of the Michigan Cancer Foundation, the Beginnings & Growth of Detroit's Anticancer Movement. *Mich. Cancer Found. Detroit* 68 (1990).

53. Hakulinen, J., Junnikkala, S., Sorsa, T. & Meri, S. Complement inhibitor membrane cofactor protein (MCP; CD46) is constitutively shed from cancer cell membranes in vesicles and converted by a metalloproteinase to a functionally active soluble form. *Eur. J. Immunol.* **34**, 2620–2629 (2004).

54. Kesselring, R. *et al.* The complement receptors CD46, CD55 and CD59 are regulated by the tumour microenvironment of head and neck cancer to facilitate escape of complement attack. *Eur. J. Cancer* **50**, 2152–2161 (2014).

55. Cui, W. *et al.* HBXIP upregulates CD46, CD55 and CD59 through ERK1/2/NF-κB signaling to protect breast cancer cells from complement attack. *FEBS Lett.* **586**, 766–771 (2012).

56. ME-180 ATCC ® HTB-33™ Homo sapiens cervix; derived from met. https://www.atcc.org/products/all/HTB-33.aspx.

57. Cui, W. *et al.* miRNA-520b and miR-520e sensitize breast cancer cells to complement attack via directly targeting 3′UTR of CD46. *Cancer Biol. Ther.* **10**, 232–241 (2010).

58. Tan, J. R. *et al.* MicroRNAs regulating cluster of differentiation 46 (CD46) in cardioembolic and non-cardioembolic stroke. *PLOS ONE* **12**, e0172131 (2017).

59. K-562 ATCC ® CCL-243™ Homo sapiens bone marrow chronic myel. https://www.atcc.org/products/all/ccl-243.aspx.

60. Raji ATCC ® CCL-86™ Homo sapiens lymphoblast Burkitt's lymph. https://www.atcc.org/products/all/CCL-86.aspx.

61. 293T ATCC ® CRL-3216™ Homo sapiens embryonic kidney. https://www.atcc.org/products/all/crl-3216.aspx.

62. Londono, C., Osorio, C., Gama, V. & Alzate, O. Mortalin, Apoptosis, and Neurodegeneration. *Biomolecules* **2**, 143–164 (2012).

63. Gregory, P. A. *et al.* The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat. Cell Biol.* **10**, 593–601 (2008).

64. Maciorowski, Z., Chattopadhyay, P. K. & Jain, P. Basic Multicolor Flow Cytometry. *Curr. Protoc. Immunol.* **117**, (2017).

65. Nucleotide BLAST: Search nucleotide databases using a nucleotide query. https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome.

66. Ganger, M. T., Dietz, G. D. & Ewing, S. J. A common base method for analysis of qPCR data and the application of simple blocking in qPCR experiments. *BMC Bioinformatics* **18**, 534 (2017).

67. Xiao, S., Shiloach, J. & Betenbaugh, M. J. Engineering Cells to Improve Protein Expression. *Curr. Opin. Struct. Biol.* **0**, 32–38 (2014).