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Expression Profiling of Non-Coding RNA by Environmental Interactions in Innate Immunity

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EXPRESSION PROFILING OF NON-CODING RNA BY ENVIRONMENTAL INTERACTIONS IN INNATE IMMUNITY

By

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An Abstract of Thesis Presented
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Cystic fibrosis (CF) is a genetic disorder that affects 30,000 people in the United States and currently has no cure. Although CF affects all of the body's systems, it is largely characterized as a lung disease. CF is characterized by a mutation in both copies of the gene for cystic fibrosis transmembrane conductance regulator (CFTR). A mutation in the CFTR gene leads to improper movement of chloride ions and water into the airways, which dysregulates the airway surface liquid volume and composition. Individuals with CF are prone to lung infections due to inefficient bacterial clearance and by the age of 18, eighty percent of patients with CF harbor *Pseudomonas aeruginosa*, with it being the most prevalent respiratory microorganism at that age.

P. aeruginosa is a gram negative, opportunistic bacterium that is found all over the planet. It is a serious threat to people with CF due to its ability to form large colonies

known as mucoid *pseudomonas.* The mucoid strain of *P*. *aeruginosa* provides increased resistance to not only antibiotics but also opsonization and phagocytosis. Even if *P*. *aeruginosa* infection can be managed there is a delicate balance between controlling the infection and causing exacerbated neutrophilia and tissue damage due to increased inflammation. Many factors can affect the balance of the immune system including environmental toxicants such as arsenic.

Arsenic exposure is a health risk to many people worldwide through contaminated drinking water. Arsenic is able to leech out of granite and other sediments into groundwater and subsequently become included in drinking water. Arsenic has been shown to affect the immune response to pathogens, which can potentially lead to increased *P*. *aeruginosa* infection and is therefore a complication in people suffering from CF.

It is important to research the effects of CF, *P*. *aeruginosa*, and arsenic to understand how they are modulation the immune system and to potentially discover novel therapies to combat CF and bacterial infection. The Kim lab recently completed RNA sequencing analysis to measure the dysregulation of genes in response to these factors in zebrafish embryos. To understand how the immune system is being modulated by these factors the role of non-coding RNAs (ncRNA) were investigated. ncRNA have remained largely uncharacterized and, until recently, were thought to serve little to no purpose in our genome. Recent studies have shown ncRNA to be involved in many important biological processes such as embryonic development, transcriptional regulation, apoptosis, and immunity. The Kim lab is particularly interested in the role of micro-RNAs (miRNA) and long intergenic non-coding RNAs (lincRNAs).

In order to better understand and characterize ncRNAs it is critical to study their mechanisms in vivo. The zebrafish (*Danio rerio*) is regarded as an excellent research model, because of the ease with which it can be genetically manipulated, the availability of transgenic lines, and its sole reliance on the innate immune system during the first 4-6 weeks of development. In addition, the zebrafish's optical clarity allows for the use of fluorescently-labeled bacterial strains to track and image infection in live zebrafish embryos. With the use of the zebrafish and high-throughput sequencing, the lab aims to aid in the discovery of novel therapies for people suffering from cystic fibrosis and bacterial infection.

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I would like to dedicate this thesis in memory of my grandmother, Nancy “Mimi” Patterson. With your memory no dream is too big.

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CHAPTER 1

INTRODUCTION

1.1 Cystic fibrosis

Cystic fibrosis (CF) is the most common genetic disease among the Caucasian population, yet still remains an orphan disease. CF affects over 30,000 individuals in the United States alone with another 40,000 worldwide. Although CF is rare it is hypothesized that one in every 31 Americans is a carrier of the disease, which means that they are asymptomatic but are able to pass it on to their children (1).

1.1.1. History

CF was not characterized until 1938 when Dr. Dorothy Anderson noticed cysts and fibrosis in the pancreas of patients, which differentiated CF from other gastrointestinal disorders (2). The cause of CF remained elusive until the summer of 1949, when it was discovered that several heat stroke patients had higher than normal concentrations of salt on their skin. It was therefore postulated that these individuals were suffering from severe dehydration and were failing to retain salt as well as healthy individuals (3). Since this discovery, physicians have been able to diagnose CF from infancy based on skin salt concentrations that exceed 60 mM.

In 1989 the gene responsible for CF was cloned and discovered by Dr. Lap-Chee Tsui and his team and identified as the cystic fibrosis transmembrane conductance regulator (CFTR) (4). The CFTR gene is located on the long arm of human chromosome 7, region q31-q32 and its gene codes for the 1,480 amino acid CFTR protein (5). The

CFTR protein is a large glycoprotein and consists of two-membrane spanning regions along with a cytoplasmic regulatory R domain. The CFTR protein is a cAMP-regulated chloride ion channel important in chloride conductance and the regulation of salt secretion from most epithelial cells. In healthy individuals CFTR secretions are thin and watery, but with the CFTR mutation they become much thicker. The thickness is due to the retention of water by the epithelial cells due to improper transport of chloride ions (1, 6).

As of today there are almost 2,000 recorded mutations of the CFTR gene, but over 70% of CF cases are due to a single amino acid deletion spanning positions 507 and 508 in the CFTR gene. This mutation ultimately results in the removal of a single codon of phenylalanine (F), which is termed p.Phe508del (also known as ΔF508 or F508del) (7). This deletion causes the CFTR protein to become misfolded during translation resulting in the partial degradation of the protein soon after generation (8). The remaining portion of the protein traffics to the cell membrane yet has reduced efficiency and function. In addition, the deletion of phenylalanine leads to a change in the coding for isoleucine at codon 507 resulting in a change from ATC to ATT. This in turn causes a structural change that reduces translation efficiency. In other CFTR mutations the protein may range from poorly regulated to completely dysfunctional, expression at lower concentrations than normal, reduced chloride conductance, poor channel gating, or the transcript may be unable to be translated. These dysfunctions are brought about by a variety of ways including: single amino acid substitutions, altered RNA processing, affected promoter regions, or large rearrangements of the gene. Whether it is a rare or

common mutation, when an individual has a defective CFTR gene in both alleles they will inevitably display a classic group of CF characteristics (1, 7).

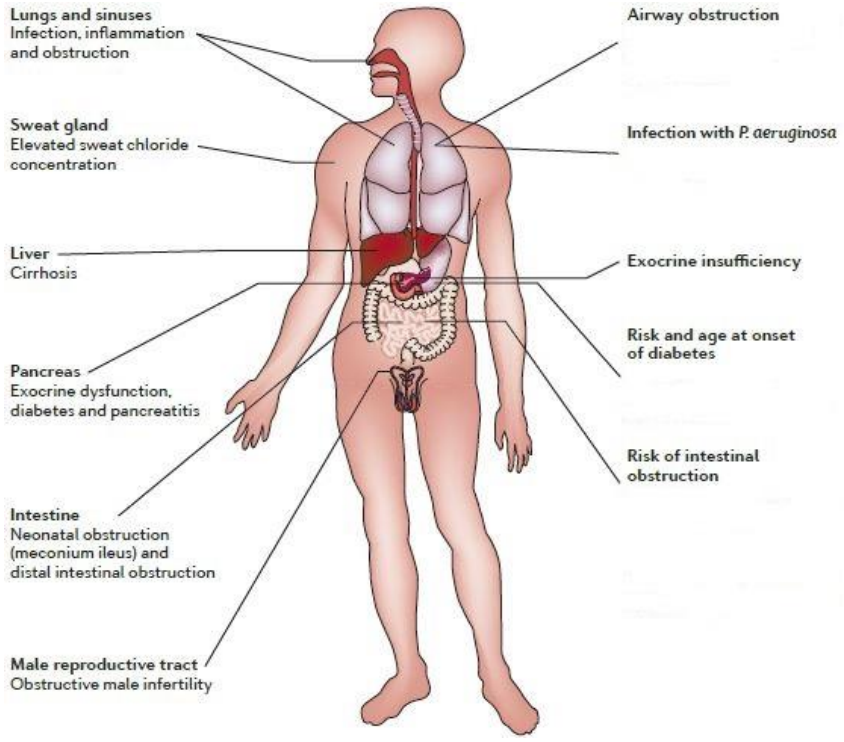


Figure 1.1. Clinical manifestations of cystic fibrosis. Cystic fibrosis is a multi-organ disease that is generally diagnosed during infancy with an elevated sweat chloride concentrated above 60 mM. The degree of organ dysfunction varies considerably depending on genetic factors, age, airway obstruction, and infection with *Pseudomonas aeruginosa*. The significance of dysfunction in each individual organ will determine the degree and/or presence of its associated disorders. Adapted from Cutting, G. R. (2015). "Cystic fibrosis genetics: from molecular understanding to clinical application." Nature Reviews Genetics **16**(1): 45-56.

1.1.2 Manifestations of cystic fibrosis

1.1.2.1. General pathology

Due to the near ubiquity of CFTR protein in all cells, CF is a multisystem disease with far reaching complications. As a result of the progress made in CF therapeutics, the predicted life span of individuals with CF has risen from about 1 year in the early 1950s to 37 years today (9, 10). As the average CF patient life span has increased, many of the less problematic complications of CF have become increasingly troublesome with age.

Complications like cystic fibrosis bone disease (CFBD) have become more prevalent. CFBD is brought about by decreased absorption of vitamin D and vitamin K due to a combination of many factors. Pancreatic fibrosis and failure, in combination with the prevalence of inflammatory cytokines, results in malnutrition and poor growth and therefore lowered bone density. In addition, the use of glucocorticoid steroids to control lung infection and inflammation has led to poor bone density, which results in poor growth. This leads to a conundrum in which it is important to control lung infection but the treatments have negative effects later in the patient's life (11-13).

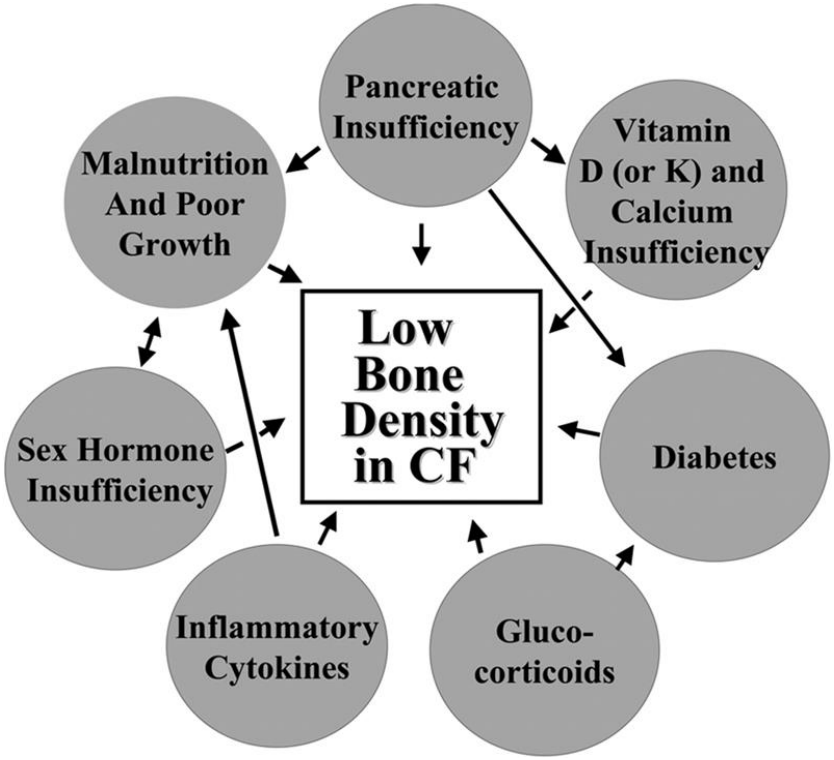


Figure 1.2. Symptoms of cystic fibrosis leading to low bone density and cystic fibrosis bone disease (CFBD). A web of complications are known to exacerbate related symptoms and lead to low bone density. Adapted from Aris, R. M., P. A. Merkel, L. K. Bachrach, et al., (2005). "Guide to Bone Health and Disease in Cystic Fibrosis." The Journal of Clinical Endocrinology & Metabolism **90**(3): 1888-1896.

As mentioned above, the pancreas is also affected by cystic fibrosis. From an early age, CFTR malfunction results in decreased transport of chloride and bicarbonate into the pancreatic ducts. This in turn causes high concentration of proteins to be secreted by the acinar cells. These reduced pancreatic secretions with high protein concentration lead to obstruction of ducts by mucous and debris, destruction of acini, and fibrosis. The fibrosis and malfunction of the pancreas over the life of the patient leads to decreased

conversion of food into energy and the regulation of blood sugar. This malfunction can cause malnutrition in the patient's early life and later in life lead to an almost complete inability to absorb fat soluble vitamins and carbohydrates. CF can eventually cause diabetes via progressively reduced levels of insulin and glucagon (14, 15).

Another major effect of CF is gastrointestinal comorbidity and complications. CFTR is exceedingly important in digestion through hydration of the mucosal lining of epithelial cells via secretion of water into the intestines. Therefore, when CF is present it can cause meconium ileus or bowel obstruction along with obstruction of the small intestine, which is a persistent problem for people suffering from CF. In fact, about 10-15% of infants with CF will die due to bowel obstruction. Recent clinical trials show that even with physician intervention, delayed hospital arrival following the rise of symptoms significantly increases morbidity (16). In addition, diseases such as celiac disease, Crohn's disease, giardiasis, and lactose intolerance are common in CF patients (3).

Although there are many nutritional deficiencies that result from pancreatic damage and gastrointestinal complications, most can be controlled through strict diets and targeted therapies. Iron deficiency (ID), however, has been very difficult to remedy and is believed to be multifaceted (17-19). ID has been long documented as an issue in 33% of pediatric CF cases and greater than 60% of adult CF cases. Interestingly, the use of iron supplements improved hypoferremia, but was unable to reverse anemia in adults with CF (20). Furthermore, in pediatric cases ID is common in healthy individuals that are properly nourished, but iron levels seem to increase with age and be positively correlated with inflammation rather than iron status (21). This suggests that something other than direct levels of iron must be the result of ID. Research has shown that the high

prevalence of ID is directly correlated with the severity of the level of lung disease in CF patients. The main factor leading to ID from lung disease is the presence of *P. aeruginosa*. Through the use of iron sequestering chemical compounds called siderophores, *P. aeruginosa* is able to sequester iron from its environment, supporting growth and proliferation. These siderophores obtain extracellular iron from the host and aid in the depletion of unbound iron in the host (18). In addition, it was found that ID is not only correlated with decreased lung function in patients with deteriorating health, but also correlated with the overall health of the patients and higher iron levels in the sputum (22). Overall, *P. aeruginosa* is responsible for ID, which is strongly associated with deteriorating health in patients with CF.

1.1.2.2. Respiratory pathology

Although CF is a multi-system disorder it is most commonly known as a lung disease, due to the overwhelming morbidity and mortality stemming from symptoms associated with CF lung disease. Roughly 80-90% of CF patients will ultimately succumb to respiratory failure as a result of defective CFTR protein (1, 23) As previously stated CFTR mutation causes a decrease in the hydration of the lung airway surface layer, causing thick mucus without salts. The thick mucus inevitably leads to impacted alveolar ducts which decreases lung function and airway dehydration. Although the use of therapeutic drugs such as corticoid steroids and mucus thinners have side effects, they have proved useful in aiding in the clearance of mucus and improving lung function. In addition, manual physical therapy and breathing techniques can be effective in aiding mucus clearance. This can be brought about though physical exercises and positioning or breathing in specific ways to loosen mucus (24, 25). Although physical and/or drug

therapies can be useful for increasing lung function, it is almost inevitable that successive bacterial lung infection will cause a tipping point which tragically cannot yet be overcome by modern medicine.

1.1.2.2.1. Respiratory bacterial infections

Chronic bacterial infections in CF patients are an unavoidable complication due to the modulation of the immune system resulting from CFTR mutation. The CF lung is the only area of the body that is exceptionally vulnerable to infections in CF patients. The urinary tract and skin, which are common areas of infection in healthy individuals, are not more vulnerable in CF patients. In healthy individuals when bacteria are inhaled the lungs will clear the pathogens without invoking a significant immune response, but the lungs of CF patients generate an excessive inflammatory response. People living with CF are unable to clear these infections easily and therefore the bacteria are able to replicate unhindered and establish chronic infections. Although the advent of antibiotics has significantly improved the life expectancy of patients with CF and is initially effective at reducing infections, antibiotics have added selective pressures which have resulted in antibiotic resistant strains of bacteria. Furthermore, many antibiotics like inhaled tobramycin are being disputed for their effectiveness and have resulted in controversy (26-28).

From childhood, bacterial colonization in CF individuals is apparent and these infections become chronic due to the host's inability to properly eliminate the infection. Although the nature of specific infections varies depending on the age of the patient, bacterial colonies are always present. *Staphylococcus aureus* and *Haemophilus influenzae*

are the most common infections reoccurring from early childhood through adolescence. These infections are commonly treated and managed with antibiotics (19, 27, 29).

Burkholderia cepacia is a complex of opportunistic pathogens comprising seven genotypically distinct species (*B. cepacia* genomovars I-VII) that are of concern to CF patients. *B. cepacia* has the ability to penetrate the thick mucus layers of the CF lung and infect the epithelial cells that are typically difficult to contact. Individuals who have reached the stage of CF lung disease that necessitates a lung transplant are also at higher risk of mortality following transplantation when infected with *B. cepacia* genomovar III pre-operation. This statistic has led many transplant centers to disallow CF patients from their transplant lists if they are colonized with *B. cepacia* (30-32). Moreover, *B. cenocepacia* may be utilizing macrophages and the innate immune system to multiply and increase mortality through the development of a fatal, pro-inflammatory response mediated by Il-1signaling (33).

Similarly to *B. cepacia*, *Stenotrophomonas maltophilia* has also been increasing in prevalence in CF patients over the past few decades. *S. maltophilia* densities increase with patient age and correlate with antibiotic exposure. The rates of *S. maltophilia* occurrence have been cited to be from 4-30% in CF patients. Broad-spectrum antibiotic treatments and selective pressure are believed to be the drivers behind the increasing incidence of *S. maltophilia* in CF patients (2, 34, 35). Like *B. cepacia*, chronic *S. maltophilia*, is also responsible for an increased risk of death or lung transplant in individuals with CF (32).

Pathogens are known to infect individuals with CF, but the most common infection involves the opportunistic pathogen, *Pseudomonas aeruginosa*. *P. aeruginosa* has been known to be the most important pathogen to infect CF patients since infections were described in the CF literature. *P. aeruginosa* is responsible for about 80% of the chronic respiratory infections in CF patients due to myriad factors including its resilience to antibiotic clearance (1, 36).

1.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram negative rod and an opportunistic pathogen in humans. *P. aeruginosa* is found in both plants and animals and due to its ability to live on many different sources of carbon, it is found all over the world in every environment humans inhabit. In healthy individuals *P. aeruginosa* infiltration rarely results in infection and is cleared without the initiation of a significant immune response. *P. aeruginosa* is also a common nosocomial infection representing roughly 10% of all nosocomial infections, is almost always associated with compromised immunity. *P. aeruginosa* owes this high pathogenicity to its effective virulence factors, such as drug pumps and other antibiotic-combating proteins. In addition, the cell membrane of *P. aeruginosa* is capable of blocking many drug targets (37). Furthermore, *P. aeruginosa* is very effective at forming biofilms on medical devices such as ventilators and catheters, rendering them even more resistant to antimicrobials and providing them with a direct route for infection. In fact, pathogens like *P. aeruginosa* have prompted hospitals to implement continence training in elderly patients and intermittent catheterization in patients with spinal cord injury (38). Also, this growing health concern has prompted the use of polymers and other compounds to coat instruments and surfaces to combat the

arms race between antibiotics and antimicrobial resistance (39). Taken together, these factors make *P. aeruginosa* a potentially dangerous microorganism, especially in hospitals where there are likely to be greater numbers of immunocompromised patients.

1.2.1 *P. aeruginosa* infection and cystic fibrosis

Due to the reduction of water transport in the lungs, the airways become dehydrated in CF patients. CFTR dysfunction leads to a lack of transport of chloride and water causing a dehydrated airway epithelium and obstructed mucociliary clearance. This in turn makes the airway a good environment for bacterial infection by *Staphylococcus aureus* or *Pseudomonas aeruginosa.* When healthy individuals inhale microorganisms, these generally get stuck in the mucosal lining of the lung epithelial cells and are unable to penetrate the mucous. In addition, the mucus is moved out of the lungs via the mucociliary elevator, which is normally very effective at keeping the lungs a largely sterile environment (2).

P. aeruginosa is the most prevalent microorganism in individuals with CF with at least 85% of CF patients harboring the pathogen. This is important because over 90% of CF patients succumb to respiratory failure (6, 40). Although standard methods for treating bacterial infection can be effective, *P. aeruginosa* frequently mutates into a mucoid form with abundant alginate and PsI exopolysaccharide production, resulting in increased resistance, not only antibiotics, but also to opsonization and phagocytosis (41, 42). This in turn causes an intense immune reaction yielding increased chemokine and cytokine expression and polymorphonuclear leukocyte (PMN) migration. In addition, serine proteases are released, causing increased inflammation and a self-perpetuating

cycle of lung damage. This increased inflammation does not correlate with decreased bacterial burden (43, 44). The obstruction of the airways eventually leads to decreased lung function, which is why most individuals with CF die prematurely from lung-related problems.

1.2.2. Virulence factors

Some strains of *P. aeruginosa* have over 15 genes coding for virulence factors, including genes that act to enable formation of biofilms, inhibit cytochrome C, and sequester iron. The roles of virulence factors in infection, and particularly infection in CF patients, have been studied extensively in an effort to understand why *P. aeruginosa* is so deadly to individuals with CF. There is no single virulence factor that can be targeted to remedy infection, but a collection of factors promote chronic *P. aeruginosa* infection (45).

Quorum sensing (QS) is part of a multicomponent communication and regulatory network that is necessary for the production of virulence factors in *P. aeruginosa* populations. QS was first observed in *Vibrio fischeri* where bioluminescence is dependent on population density. *P. aeruginosa's* QS network involves two independent QS systems, LasIR and RHIIR (45). Biofilm formation is also a result of QS, which begins when signal molecules 3-oxo-C12-homoserine lactone (HLS) and C4-HLS reach high enough concentrations to trigger Las and Rhl transcription factors as a result of high bacterial densities. Once Las and Rhl are triggered and activated a signaling cascade ultimately results in the formation of *P. aeruginosa* biofilms (46-49).

Moreover, it has been proposed that even though QS is a pivotal player in the initial stages of infection and biofilm formation, its role may be less important over the duration of the infection (50). Indeed QS is important initially, with recent results showing that blocking pyocyanin and biofilm formation protects *Caenorhabditis elegans* and human lung epithelial cells from death caused by *P. aeruginosa* (51). Later in the timeline of chronic infection QS appears to become less important in that *P. aeruginosa* isolates from 166 CF patients were LasR mutants. This study suggests that LasR mutations may even be selected-for and correlated with the severity of lung disease (52). Furthermore, a study that analyzed 36 *P. aeruginosa* lineages found that there was convergent molecular evolution in 52 genes, suggesting that mutation and remodeling of regulatory networks have a role in adapting to the host and the selective pressures on *P. aeruginosa* (53). Interestingly, it has been proposed that QS mutants, given the moniker "cheating bacteria", may utilize the functional QS systems of other members of the population as long as a balance is maintained (54-57). Taken together, it is apparent that the QS system is essential for early biofilm formation of *P. aeruginosa*, but becomes less vital to chronic infection as it adapts to selective pressures form the host.

1.2.2.1. Secretion systems

P. aeruginosa has distinct secretion systems that based upon their substrates have a spectrum of functions (58). Type I secretion system (T1SS) and type II secretion system (T2SS) transport various molecules through the inner and outer membranes of the bacteria to the outside the cell (59, 60). The type III secretion system (T3SS) acts as a molecular syringe to secrete effector proteins directly into the eukaryotic cell from the

bacterial cell (60, 61). These three systems act to release virulence factors and thereby aid in the pathogenicity of *P. aeruginosa*.

T1SS in *P. aeruginosa* mainly involves the function of alkaline metalloproteases (aprA) to degrade complement and cause hydrolysis of fibrin. T2SS virulence factors include: elastases (lasA and lasB), proteases (prpIL), and lipases (plcB), which, in combination with aprA, play a critical role in *P. aeruginosa* pathogenesis (62). Although these virulence factors have long been known to act in a plethora of ways on a variety of targets, they are all important components of *P. aeruginosa* pathogenesis (63, 64). In addition, these factors have also all been implicated in lung disease and CF (65-67). Furthermore, these virulence factors are regulated by the QS system through transcription factors Las and Rhl (47, 49, 68).

The T2SS also secretes the most toxic of the *P. aeruginosa* virulence factors: Exotoxin A (toxA). toxA is regulated by QS and is a member of the mono-ADP-ribosyltransferase family, which inactivates eukaryotic elongation factor 2 (eEF-2) by adding an additional ADP-ribose moiety to eEF-2 in a reaction called ADP-ribosylation (69, 70). Without eEF-2 the epithelial cells will necrotize from the lack of protein synthesis (71, 72). toxA has been shown to adapt both structurally and functionally during the course of infection to retain the pathogenicity of *P. aeruginosa* (53). toxA has been studied extensively and is thought to be a critical component of *P. aeruginosa* infection and degeneration of lung health in CF patients (68, 73, 74).

T3SS in *P. aeruginosa* is used to directly inject exotoxins into its host's lung epithelial cells. T3SS in *P. aeruginosa* delivers four different exotoxins, which include

exotoxin S (exoS), exotoxin U (exoU), exotoxin T (exoT), and exotoxin Y (exoY) (61, 75). ExoS and exoT are closely related and are members of the mono-ADP-ribosyltransferase family, which inactivate eEF-2. In addition, exoS and exoT have an N-terminal GTPase activating protein that targets the Rho family of GTPases, which are molecular switches involved is many common cellular functions. ExoU has phospholipase A2 which degrades the plasma membrane of cells to cause cell death (76). The activity of ExoY is not completely understood, but it is known to cause inter-endothelial gaps and vascular leak, which in turn lead to inflammation and exudation (77, 78). T3SS is well characterized as a potent system necessary for infection in a wide variety of flora and fauna (76, 79, 80).

1.2.2.2. Siderophores

In the case of *P. aeruginosa*, iron acquisition is essential for infection. Iron is normally bound tightly to transferrin or in the case of airways, lactoferrin (81-84). *P. aeruginosa* accomplishes this via two major siderophores: pyochelin and pyoverdin. The acquired iron is essential to many of the metabolic processes in *P. aeruginosa* and is involved in the regulation of other virulence factors, such as exoA, proteases, and even pyoverdine (81, 85, 86). Interestingly, it has been proposed that iron chelators could be used as drugs to treat patients with CF. Iron chelation drugs could have a dual effect of preventing *P. aeruginosa* from acquiring iron and dampening the inflammatory response of neutrophils by reducing the production of reactive oxygen species. In the absence of iron, reactive oxygen species (ROS) would be dampened due to the major role that iron plays in its generation (87).

1.2.2.3. Biofilms and mucoid *Pseudomonas*

P. aeruginosa biofilms that form in the lungs of CF patients pose a deadly threat. *P. aeruginosa* biofilms present problems for immunocompromised patients, as *P. aeruginosa* commonly forms biofilms on hospital equipment such as catheters and respirators. These biofilms are bacterial communities directed by the QS system, which leads to virulence factor production and increased antibiotic defense (57, 88).

In almost all cases of biofilm formation in CF patients, *P. aeruginosa* eventually mutates into a mucoid form that is not found in non-CF patients (89). Mucoid *Pseudomonas* microcolonies are enclosed within a fibrous anionic matrix making them further resistant to opsonization, phagocytosis, and antibiotics (41, 90-92). The emergence of mucoid *Pseudomonas* has been correlated with the evolution of antibiotic resistance in CF patients (89, 93).

Mucoid *Pseudomonas* bacteria are commonly located in hypoxic masses in the airway lumen. These infections are known to penetrate the hypoxic mucus zones and respond with the production of alginate, which enhances adhesion (94). Growth in these hypoxic environments further increases hypoxia, which in turn produces increased anaerobiosis (95). When the research is taken together it illustrates the need for novel therapies to prevent and/or destroy mucoid *Pseudomonas*. The bulk of current research in *Pseudomonas* pathogenicity is focused on this problem (87, 96-100).

1.2.2.4. Pyocyanin

Pyocanin (N-methyl-1-hydrophenazine) is a toxin produced by *P. aeruginosa* colonies that is responsible for the characteristic blue-green color of *P. aeruginosa*

colonies. Exposure to high concentrations of pyocanin can result in many serious complications including: inflammation, disruption of lung airway epithelium, and inhibition of lung ciliary motility. In addition, pyocanin also inhibits catalase, which then leads to increased oxidative damage. In combination with the over-activity of PMN ROS production, the absence of catalase function can be devastating to the lungs of CF patients (45, 49, 86, 101).

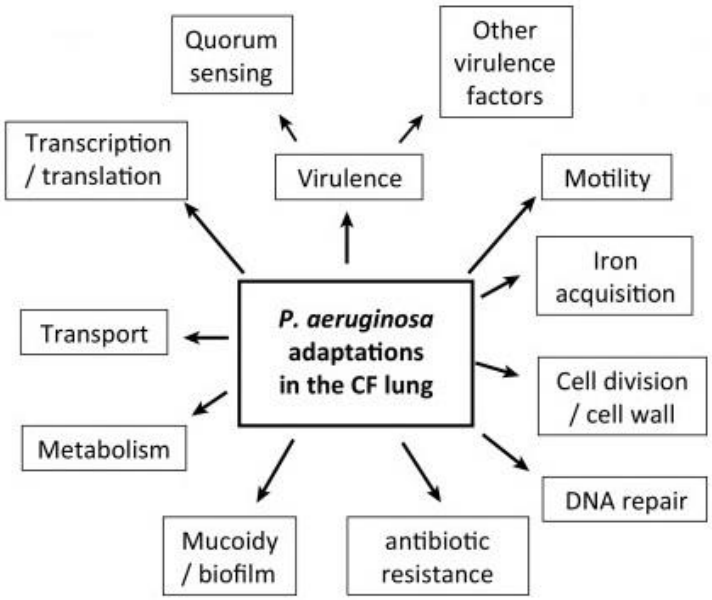


Figure 1.3. Adaptations of *Pseudomonas aeruginosa* in the lung of individuals with cystic fibrosis. *P. aeruginosa* is the major cause of death in individuals with CF. It is a highly versatile gram-negative bacteria that employs a plethora of virulence factors leading ultimately to the formation of mucoid *Pseudomonas*. Mucoid *Pseudomonas* is largely responsible for the chronic and persistent lung infections that are highly resistant to both immune and antimicrobial therapy clearance. Adapted from Winstanley C, O'Brien S, Brockhurst MA. 2016. Pseudomonas aeruginosa Evolutionary

Adaptation and Diversification in Cystic Fibrosis Chronic Lung Infections. Trends in Microbiology **24:**327-337.

1.3. Innate immunity

1.3.1. Overview

The innate immune system is responsible for distinguishing self from non-self and is also the body's first line of defense which clears infections through a variety of methods. Understanding the function of the innate immune system, or rather its dysfunction in CF patients, is critical for developing effective therapies. Although CFTR is not normally associated with immune health in healthy individuals, it plays a major role in the immune response in CF patients.

1.3.2. Pattern recognition receptors

Pattern recognition receptors (PRRs) are used by the innate immune system to identify pathogens by their pathogen-associated molecular patterns (PAMPs). PAMPs are made of foreign moieties such as proteins and lipids that are similar within a group of pathogens. PRRs reside on the surface of innate immune cells and recognize PAMPS to trigger a signaling cascade. Important PRRs for bacterial infections in CF patients include Toll-like receptors (TLRs) 2, 4-6, and 9. TLRs have been extensively studied and each is known to recognize specific PAMPs that are specific for different classes of bacterial, fungal, and viral pathogens. TLRs will recognize specific moieties including: triacyl lipopeptides, lipopolysaccharide (LPS), flagellin, and specific CpG motifs. For example, TLR-2 is known to be driven by lipoproteins and peptidoglycan from gram-positive

bacteria like *S. aureus*, while TLR-4 is important for the recognition of gram-negative bacteria such as *P. aeruginosa*. In CF patients, TLR4 in the epithelial lung cells is reduced, resulting in decreased activation of MyD88 signaling and Trif signaling, both of which are involved in pathways linked to type I interferon signaling (INFs) (102). INF signaling is known to be necessary in the clearance of multiple lung-associated pathogens (103). Interestingly, it has also been shown that CFTR itself is a PRR specific for the O-side chain of *P. aeruginosa* LPS and is essential to effectively coordinate an innate immune response to *P. aeruginosa* (104-106). Once a PRR recognizes a PAMP it will trigger a signaling cascade, which will result in many downstream actions including: the induction of inflammatory signaling, vasodilation, temperature changes, and phagocyte recruitment. The recognition and subsequent response to bacterial infections in the CF lung is essential to the health of CF patients (23, 107).

1.3.3. Cytokines and inflammation

At an early age, even before apparent infection, there is evidence of inflammation in the airways of people with CF via excessive, defective PMN invasion and overly abundant concentrations of cytokines, cytotoxic granules, and free proteases (108). The chronic invasion of dysfunctional PMNs yields a constant activation of NF-κB signaling resulting in drastically elevated levels of reactive oxygen species (ROS). Therefore, concentrations of pro-inflammatory cytokines (IL-8, IL-6, IL-1β, and TNFα) are significantly increased in addition to significantly decreased expression of the anti-inflammatory cytokine IL-10. In healthy lungs, CFTR aids in the moderation of NF- κB signaling during oxidative stress by managing the degradation of nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor, α (I-κB-α), but in the CF lung this process

is dysfunctional due to decreased CFTR function (109). In addition, CFTR mutation leads to diminished trafficking of the antioxidants gluthathione (GSH) and thiocyanate (SCN^-). Without the proper movement of these antioxidants, oxidative stress alters IL-8 expression and decreases *P. aeruginosa* clearance (110).

1.3.4. Immune cells

Innate immune cells are essential in the clearance of pathogens and quickly respond to wounding and infection. Innate immune cells include a wide array of cell type including mast cells, natural killer cells, and phagocytes. The most immediately responding of the innate immune cells are the phagocytes and in particular the neutrophils and macrophages. These cells are important in innate immunity because they act to phagocytize invading pathogens and destroy them intracellularly through the use of specialized compartments with low pH called lysosomes. When a pathogen is phagocytized it enters a phagosome, which will be subsequently merged with a lysosome to form a phagolysosome. Inside the phagolysosome the pathogens are destroyed via a combination of low pH and reactive oxygen species (ROS) (111). Phagocytes like the neutrophil can phagocytize and destroy up to 20 bacteria per cell in rapid succession (112).

1.3.4.1. Respiratory burst

An important method employed by phagocytes is the respiratory burst response, which releases ROS into the extracellular environment. PMNs and macrophages produce the majority of ROS, which includes hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), hypochlorous acid (HOCl), and hydroxyl radicals (OH^-). ROS are released as a result of

phagocytes attempting to destroy engulfed pathogens. The major source of ROS as a result of phagocytes is generated by NADPH oxidase (NOX). Once NOX is activated, its subunits will be phosphorylated and translocated to the membrane of the phagosome where NOX is located. This allows the subunits to associate with the membrane subunits of NOX, which will assist in generation of free oxygen radicals by reducing O_2 to O_2^- (113, 114). A dismutation reaction will then follow to produce H_2O_2. Myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS) are also important in the generation of ROS by producing HOCl and nitric oxide (NO^-) (115, 116). These two molecules can then react with superoxide to form OH^-. iNOS expression has been shown to be decreased in CF patients and is implicated in their susceptibility of bacterial infections (27, 117-120).

Previous literature has shown that too much ROS can cause accidental, collateral damage, but too little can lead to pervasive and/or chronic infections (117). Moreover, the cells that produce ROS can also lead to many diseases like localized lung injury, acute respiratory distress syndrome, asthma, and COPD (121). The literature has shown that knocking down *ncf1*, a subunit of NOX, will lead to complete knockdown of NOX and subsequent ROS production in phagocytic cells. NOX has been found to be essential in the clearance of fungus and continued recruitment of phagocytes. Using time-lapse imaging Brothers et al. showed that NOX was essential in promoting the continued chemotaxis of macrophages to the site of a *Candida albicans* fungal infection (122). Alternatively, it has been shown that knock down of NOX has led to decreased lung tissue damage, reduced inflammation, and decreased viral burden in mice infected with influenza (121). Furthermore, some individuals are born with a congenital

immunodeficiency called chronic granulomatous disease (CGD), which is characterized by a defect in the production of ROS. These individuals suffer from severe recurrent bacterial and fungal infections and extensive inflammatory reaction that can result in granulomatous lesions (123). Taken together the proper regulation of ROS in an individual can be indicative of general health, but the literature has shown conflicting results on the benefit versus injury resulting from ROS (1, 124-126). ROS are essential to the clearance of many pathogens and their continued study may prove to be important in controlling infection in CF patients.

1.3.4.2. Polymorphonuclear leukocytes and CF

Although PMN accumulation is a known problem for CF patients, how CFTR dysfunction in these immune cells plays a role in innate immunity is not well understood (127). PMN are the first responders to cites of infection and wounding and play an essential role in the clearance of pathogens, but in CF patients PMN have been shown to do more harm than good (128, 129). In fact, neutrophil production of elastase is positively correlated with decrease lung function in individuals with CF (130, 131). One confounding factor in the study of these immune cells in the CF lungs is that a significant amount of research has shown immune cells to function normally outside the lung and there are no known defects in macrophages or T cells in individuals with CF (6). Recently it has been proposed that lung PMNs may be different than PMNs found in the blood in CF patients (132-135). Moreover, PMNs in the CF lung have been shown to have increased expression of CD39, CXCR4, CD114, and RAGE, which are involved in metabolic and stress pathways (136). CF lung PMNs have also been shown to have a decreased respiratory burst (134). Controversially, PMNs in newborns with CF had no

discernable differences in function when compared with healthy controls (137). These results suggest that more research is necessary to fully understand the function of PMNs in CF.

Macrophages, neutrophils, and eosinophils have a detectable level of CFTR expression, which has been proposed in CF patients to interfere with trafficking of chloride ions for the production of bacteria killing compound HOCl, generation of the H^+ gradient across the cellular membrane, activity of NADPH Oxidase, and the control of pH in lysosomal compartments used in the destruction of phagocytosed pathogens (127, 138-142). These results have caused controversy, because an attenuated immune response should cause other organs besides the CF lung to be prone to chronic infection, but this is not the case. One explanation that has been offered suggests that it may be possible that other organs are able to overcome this immune deficit is some way that the lungs cannot (112).

Furthermore, it has been suggested that PMNs may have a reduced rate of apoptosis, explaining their prolonged residence in the CF lung. PMN defense against apoptosis may be explained by a resistance to the pro-apoptotic tumor necrosis factor α (TNF-α) and the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), which is produced in greater concentrations in CF epithelial cells compared to non-CF epithelial cells (6, 143). In addition, the presence of PMN proteases also prevents phagocytosis, which cleaves macrophage receptors and causes decreased recruitment and bacterial clearance (144). The exact role of PMN in CF is still not well understood and remains highly controversial (129).

1.3.5. Arsenic exposure

Arsenic exposure through contaminated drinking water is a health risk to many people worldwide. Arsenic is a chemical element and common contaminate in aquifers and wells, especially in areas with high levels of granite such as the northeast United States (145, 146). Arsenic leaches out of granite and other sediments into groundwater at high rates. The Environmental Protection Agency (EPA) has set a limit on arsenic concentration in well water to 10 parts per billion (ppb) (*i.e.* µg/L). Although the limit was reduced from 50 ppb to 10 ppb in 2006, it has been proposed that no level is safe. In fact, as low as 2 ppb has been shown to dampen the respiratory burst response of zebrafish and increase the load of viral and bacterial infections. In addition, arsenic exposure at 2 and 10 ppb leads to a significant decrease in antiviral and antibacterial cytokines including IL-1β and TNF-α, which can further exacerbate phagocytosis reduction already present in CF patients (147, 148). Arsenic has been shown to affect the immune response to pathogens, which can potentially lead to increased *P. aeruginosa* infection and is therefore a complication in people suffering from CF (148). In addition to the modulation of the immune response to viral and bacterial infection, environmental arsenic exposure has also been implicated in impaired CFTR function. Research has shown arsenic to increase the c-Cbl-mediated ubiquitination and degradation of CFTR in the human lung and to subsequently reduce CFTR chloride secretion, which, when defective, is known to cause decreased clearance of pathogens (149, 150). Moreover, in a small clinical study 11 patients were identified to have abnormal sweat salt concentration with no genetic diagnosis of CF. These 11 patients with high levels of arsenic in their drinking water had higher than normal sweat conductivity (151). When taken together

these studies point to arsenic contamination in drinking water as a confounding factor in CF and bacterial infections.

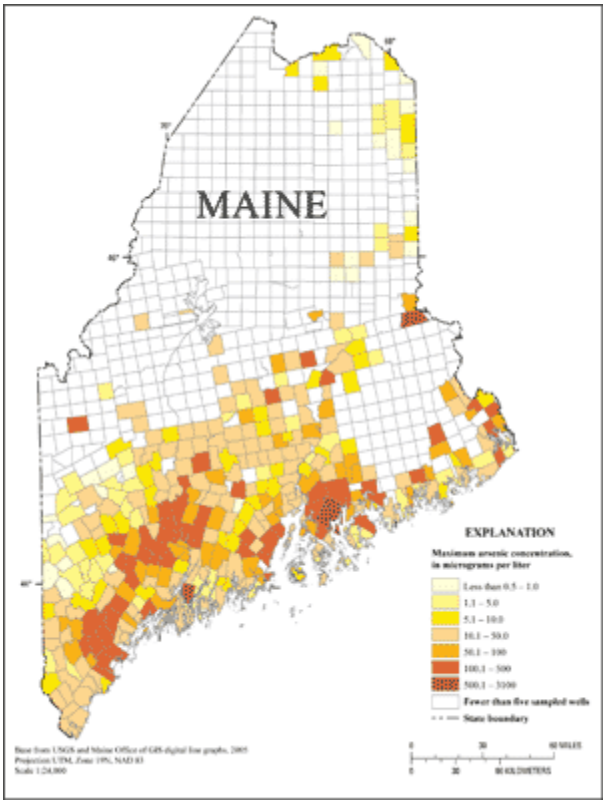


Figure 1.4. Arsenic concentration sampled from towns in Maine from 2005-2009. Approximately 10% of Maine wells have arsenic concentrations above the Environmental Protection Agency's maxium limit of 10 micrograms per liter (µg/L). Image from: Nielsen MG, Lombard PJ, Schalk LF. 2010. Assessment of arsenic concentrations in domestic well water, by town, in Maine 2005-09. Credit: US Geological Survey.

1.3.6. Non-coding ribonucleic acids

Non-coding ribonucleic acids (ncRNAs) are a large group of RNAs that are transcribed but not translated. The central dogma of molecular biology includes DNA being transcribed into RNA which is then translated into protein, but ncRNA do not initiate translation after transcription. Although ncRNA were initially thought to be little more than filler that neither increase nor decrease biological fitness, they have been studied increasingly over the past few decades. Today, ncRNAs are divided up into two main groups based on length: short ncRNAs (sRNAs) (<200 nt) such as microRNAs and long non-coding RNAs (lncRNAs) (>200 nt) (152). Both sRNAs and lncRNAs are involved in the regulation of the innate immune response (153, 154).

1.3.6.1. microRNAs

microRNAs (miRNAs) are sRNA molecules that are approximately 22 nucleotides in length and act to regulate mRNA post-transcriptionally. In short, a primary miRNA (pri-miRNA) is transcribed by RNA polymerase II and is characterized by its hairpin RNA structure. This hairpin structure is then recognized by Drosha, an RNAse III enzyme, and its cofactor DGCR8. These proteins join a complex of proteins to cleave the pri-miRNA into precursor (pre-miRNA). The pre-miRNA is then translocated to the cytoplasm with the help of Exportin-5 and Ran-GTP where it then interacts with Dicer and TRBP. Dicer and TRBP cleave the hairpin and form the miRNA duplex. The strand with the least stable 5’ end is chosen to be the guide miRNA while the opposite strand is degraded. The guide strand of the miRNA duplex will then be joined to the RNA-induced silencing complex (RISC) with the guidance of Ago2. This new complex is called the

mature miRNA, which will then bind to the 3' untranslated region of its target transcript via the guidance of the RISC. If the miRNA is perfectly complementary to its target it will initiate cleavage by Ago2 and if it is imperfectly complementary it will repress the translation of the mRNA. In addition, the mature miRNA complex can also cause mRNA deadenylation that will shorten the poly(A) tail of its target and lead to degradation (154-156). The expression of over 70 percent of all protein coding genes is regulated by miRNAs, which means that miRNAs play a role in regulation across most critical biological processes. Interestingly, one single miRNA may regulate many distinct transcripts or multiple miRNAs may regulate a single transcript.

Furthermore, miRNAs are known to play a role in a multitude of complicated human diseases including Down syndrome, Huntington's disease, cancer, and CF. miRNA-223 controls the production and activation of granulocytes and miRNA-223 ablation in mice has resulted in hypermature, hypersensitive granulocytes (157). miRNAs have been implicated in the regulation of important factors of CF including inflammatory signaling and the unfolded protein response (UPR) (158). One miRNA of interest, miR-199a-5p, is involved in the UPR in chronic obstructive pulmonary disease and may prove to be an interesting target for CF research (159).

1.3.6.2. Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are non-protein coding RNA that are greater than 200 nucleotides long, and are broken up into three categories: intergenic, intronic overlap, and antisense exonic overlap (Figure 1.5). These lncRNAs are involved in numerous biological processes including regulation of the cell-cycle, apoptosis, cell

identity, and the innate immune response (153, 160, 161). It is believed that there are more than ten transcriptional units that overlap each protein-coding gene with the vast majority of them being lncRNAs (162). There is controversy over the functionality of most lncRNA, because in most cases transcription produces short and mostly unstable transcripts that have a significantly lower level of expression compared to protein-coding genes (163, 164). This may be the case for most lncRNA, but it is believed that if even 10% were functional there would be greater than a thousand known lncRNAs that would have a role in biology (153). In fact, there have been many recent cases where functional lncRNAs are found and expressed at higher than expected concentrations (153, 164-166).

The exact mechanisms of function of lncRNAs are still being discovered, but many have been elucidated to be necessary for proper gene control across all kingdoms. In the beginning of lncRNA research it was discovered that a non-coding protein, Xist, plays an important role in the epigenetic regulation of X-chromosome inactivation (XCI). XCI is a vital process in mammals that ensures that females have only one copy of the X chromosome in each cell. Xist was found to be expressed only on the inactive X chromosome and essential for the silencing process (162, 167, 168). The discovery of Xist and its function changed the way that researchers viewed lncRNA and has led to the discovery of many novel functions and roles for lncRNA across biology. lncRNAs have also been shown to act as molecular scaffolds for organization in ribonucleoprotein complexes and chromatin states (166). Moreover, they have been found to be involved in cotranscriptional regulation, binding proteins and chromatin, nucleation of nuclear domains, pairing with other RNA, acting as molecular decoys, and more functions are believed to exist that have yet to be discovered (Figure 3.1.) (153). lncRNAs are the true

frontier in molecular biology and their continued discovery and characterization may be important for discovery of novel therapies for human diseases.

In an effort to identify noncoding RNAs and their corresponding genes more simply, most large scale analyses have focused on long intergenic (or intervening) noncoding RNAs (lincRNAs). lincRNAs have the benefit of neither having overlapping exons nor protein coding regions, nor are they antisense to a protein coding gene. lncRNAs as a whole are considered to be largely nonfunctional transcripts, but burgeoning research is finding novel functions for previously nonfunctional lncRNAs. Many large scale experiments have been conducted to catalog lncRNA loci and transcripts across many species in an effort to better understand their roles in biology (153). Zebrafish lncRNA loci and transcripts have also been cataloged, which may prove to be useful in elucidating the function of lncRNA in a relatively tractable animal model (161, 169, 170). Interestingly, like miRNAs, lncRNAs have also been implicated in CF, with many lncRNAs differentially expressed between CF and non-CF individuals and many of the lncRNAs related to inflammation in the CF bronchial epithelium cells (152). It is therefore important to continue research into the relationship between lncRNAs and inflammation in individuals with CF.

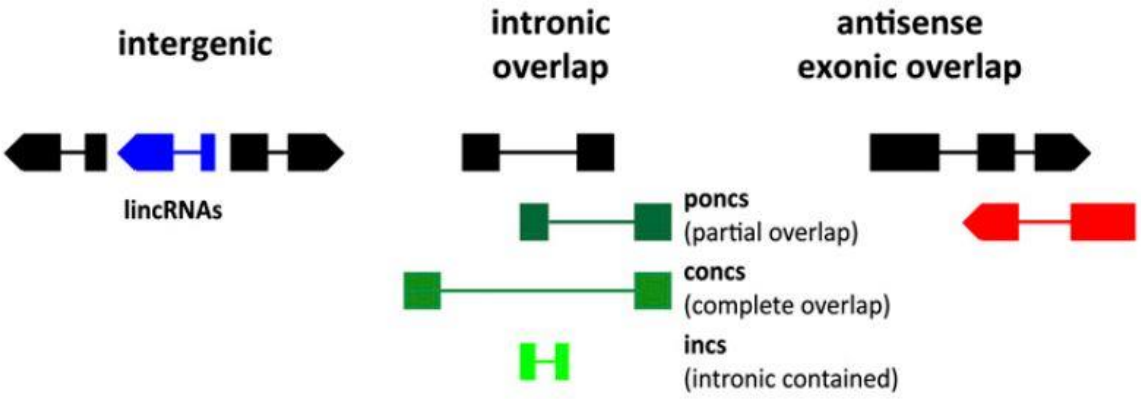


Figure 1.5. Categories of long non-coding RNA. Adapted from Pauli A, Valen E, Lin MF, Garber M, Vastenhouw NL, Levin JZ, Fan L, Sandelin A, Rinn JL, Regev A. 2012. Systematic identification of long noncoding RNAs expressed during zebrafish embryogenesis. Genome research **22:**577-591.

1.4. Zebrafish as a model organism

The zebrafish (*Danio rerio*) is a model that has become increasing popular in the past few decades, due initially to an effort to reduce, replace, and refine animal models used in research. Though what initiated the use of the zebrafish as an animal model is still important today, the uniqueness of the model is what has brought them into prominence. The zebrafish model has found its own niche in animal research due to a many beneficial characteristics, including high fecundity and production of young *ex utero,* which allows simplified injection with a large sample size. Moreover this allows for high throughput sequencing and analyses. Furthermore, the zebrafish's rapid development allows for the creation of all of their primordial organs within the first 24 hours post fertilization. In addition, their cost is low and maintenance is relatively simple, compared to many other

popular models such as the mouse. In a single facility thousands of fish can be maintained by a single technician. The optical transparency of zebrafish allows for visualization and imaging of infection with fluorescent pathogens and/or immune cells in vivo and in real time, which is not possible in other nontransparent animal models (171-173). Zebrafish are also genomically and physiologically similar to mammals (174, 175). Importantly, there are many genetic tools available for the zebrafish, which allows researchers to study the immune system and host-pathogen interactions in a way that cannot be replicated in mammalian models or cell culture (171).

In addition to the many benefits of using zebrafish in research, they also have an immune system that is highly homologous to that of humans and haven been characterized in the context of many human diseases and infections including CF (176-178). At 48 hours post fertilization the zebrafish has a functional innate immune system and until about 4 to 6 weeks post fertilization they are without an adaptive immune system (172). This distinction of time between the maturation of the two immune systems allows researchers to study the innate immune system either alone or in combination with the adaptive response. The components of the immune system are also highly conserved between zebrafish and human, with zebrafish expressing an almost complete set of toll-like receptors in addition to cytosolic sensors and signaling molecules, which allows researchers to target specific pathways and systems. Although the intermediates of some of these pathways may be different, the functionality is conserved (173).

Zebrafish are also susceptible to bacterial, viral, and protozoan infections including *P. aeruginosa*, which are managed by the immune system in much the same way as the human immune system. Zebrafish are susceptible to P. *aeruginosa* and

respond to the infection by upregulating many characteristic cytokines such as IL-1, TNF-α, and CXCL8L1/L2 (IL-8). Leukocytes like neutrophils and macrophages also respond to infection immediately following viral, bacterial, and fungal infections (122, 177).

Recently the zebrafish was established as a model for CF and *P. aeruginosa* infection. It was found that CFTR morphant zebrafish have a reduced respiratory burst, decreased neutrophil migration, and decreased clearance of *P. aeruginosa*. In addition, it was shown that zebrafish challenged with other common respiratory infections in CF patients, did not show the same resistance to bacterial clearance that was see in *P. aeruginosa* infected zebrafish. The LasR QS system of *P. aeruginosa* was found to be responsible for the zebrafish's reduced ability to clear the infection (177). When taken together, the zebrafish is a good research animal to model the innate immune response to *P. aeruginosa* in a CFTR morphant system.

The zebrafish genome is greater than 70% homologous to human and is completely sequenced, which allows for the use of targeted genetic manipulation with the use of tools like clustered regularly interspaced short palindromic repeats (CRISPR), zinc-finger nucleases (ZFNs), and transcription activator-like nucleases (TALENs), and morpholino oligonucleotides (MOs). CRISPR is an important tool for manipulation available to zebrafish researchers. The CRISPR system is a powerful tool that is natively found in bacteria and archaea as a defense mechanism. This system adapts by incorporating sequences derived from foreign pathogens into a small-RNA-based repertoire. This repertoire is then able to prime a complex of proteins to recognize and destroy invading pathogens (179). In zebrafish research this can be used as a means to

knock-out target genes by customizing single guide RNAs (sgRNAs) that can direct Cas9 to endogenous zebrafish genes. Cas9 is an endonuclease that can cause site-specific DNA cleavage, which can therefore knock-out targeted zebrafish genes (180).

ZFNs can also act similarly to CRISPR by targeting specific loci in zebrafish and disrupt targeted genes. ZFNs act to target a specific locus and cause a double-break in the DNA that is subsequently repaired. During the repair process small insertions and deletions are generated. When ZFNs were designed and injected into zebrafish embryos to target the *no tail/Brachyury* (*ntl*) gene, characteristic mutations were observed. Unlike CRISPR, which completely knocks-out targeted genes, ZFNs were only able to disrupt ntl and cause a loss of function is about half of the injected embryos with an average function of 20% (181). This approach, therefore, is used less often with the advent of the CRISPR/Cas9 system. In addition, the engineering of ZFNs has proven to be challenging due to the necessity to account for the effects of each individual "finger" in the zinc-finger nucleases (182).

Another useful system for genome editing in zebrafish are TALENs. TALENs are transcription activator-like effector repeats fused to the nonspecific Fokl cleavage domain that are constructed to influence specific double-stranded breaks in cells. TALENs are very efficient and are capable of altering the genes at or near the site of the double-stranded break. TALENs are also relatively easy to engineer and can be formulated to target most sequences (182, 183).

Morpholinos are antisense oligonucleotides that are able to bind to complementary RNA and either prevent transcription or modify RNA splicing and are therefore able to knock-down specific genes such as the *cftr* gene. Morpholinos can be injected into the embryos at the one-cell stage, which will effectively knock-down the targeted gene in every cell for a limited duration, usually remaining effective for 3-4 days post injection (184).

One important genetic change that has been made to zebrafish is the establishment of a transgenic zebrafish line, tg(MPX:GFP). This line expresses GFP under the control of the promotor for the neutrophil-specific-myeloperoxidase (MPX), which yields green fluorescent neutrophils. This line of transgenic zebrafish has been instrumental in investigating the inflammatory response of neutrophils to wounding and infection, as well as characterizing their migration (185, 186). A crucial aspect of neutrophils, among other granulocytes, in the innate immune response is the production of a respiratory burst which results in the release of ROS. ROS have an important role in clearing invading pathogens after an infection. Another benefit to the zebrafish as a research model is the relative ease of measuring ROS via the recent development of cheap, efficient assays. In short, zebrafish are exposed to reduced 2'7'-dichlorodihydrofluorescein diacetate and phorbol 12-myristate 13 –acetate (PMA). PMA is used to activate protein kinase C and subsequently induce a respiratory burst from immune cells. During the assay the 2'7'-dichlorodihydrofluorescein diacetate is oxidized and becomes fluorescent, and can therefore be measured with a standard plate reader (187, 188).

The many beneficial characteristics of the zebrafish combined with its susceptibility to *P. aeruginosa* and the ability to knock-down the CFTR gene, make it an excellent animal model for the study of the role of ncRNA in innate immunity and environmental interactions. In addition, the availability of cataloged lincRNA loci and transcripts allows researchers to investigate the roles of specific lincRNAs to potentially elucidate the roles in immunity. It is evident that the zebrafish is a powerful animal model for research into immunity.

CHAPTER 2

KNOCKDOWN OF MIRNA-199 IMPROVES RESPONSE TO INFECTION BY *P. AERUGINOSA*

2.1. Introduction

Cystic fibrosis (CF) is a multi-organ orphan disorder that is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). CFTR is a gene that codes for a chloride ion channel that is expressed in epithelial cells, lymphocytes, polymorphonuclear (PMN) cells, and many more cell types, and is responsible for the regulation of ion transport. When CFTR is mutated it causes a loss of airway surface liquid from improper transport of ions, which results in thick mucus formation. The formation of thick mucus, along with poor pancreas function leads to the bulk of the clinical manifestations of CF, including digestive issues, malnutrition, and improper clearance of lung infections (6). Although CF is a multi-organ disease, the majority of CF patients will succumb to lung infections, with *P. aeruginosa* being the most common pathogen in adult CF patients (23, 49). Antibiotics and other therapies are initially effective for improving lung function, but successive infections and myriad *P. aeruginosa* virulence factors and biofilm/mucoid formation leads to an infection that cannot yet be overcome by modern medicine.

Much effort has been put into developing novel therapies to treat and even cure people with CF, but at this time nothing can prevent the almost always fatal lung infections. To better understand CF and the role that *P. aeruginosa* is playing in immunity, the zebrafish model has been employed. Zebrafish (*Danio rerio*) is a member

of the teleost family of vertebrates and has become an excellent animal model for the study of many human diseases and infections. The zebrafish is genomically and physiologically similar to humans and utilizes its innate immune system solely for the first 4-6 weeks of life (172, 174, 175). The distinction between innate and adaptive immunity, combined with the availability of genetic tools, efficient assays, translucent body, high fecundity, and relatively low cost of care and maintenance allows researchers to study the immune system and host pathogen interactions in a way that could not be replicated as effectively in other animal models or cell culture (171).

Recently, the zebrafish has been shown to be a good animal model for CF knockdown (KD) and *P. aeruginosa* infection (177). Phennicie et al. found that CFTR KD via morpholino oligonucleotides (MO) in wildtype (wt) zebrafish leads to a dampened respiratory burst and when infected with *P. aeruginosa* the zebrafish displayed a statistically higher bacterial burden in the CFTR KD embryos when compared to control embryos. In addition, the migration of neutrophils was found to be decreased in the CFTR KD zebrafish compared to the control zebrafish, which could potentially explain the decreased respiratory burst and consequently higher bacterial burden (189).

In addition to the major problem of *P. aeruginosa* infection in CF patients, arsenic is another factor that has potential to be dangerous to CF patients living in areas with high concentrations of this element in well water. Arsenic has recently been shown to degrade CFTR and to subsequently reduce chloride secretion, thus exacerbating CF (149). In addition, arsenic concentration has been correlated with increased sweat chloride conductivity in a small clinical trial of non-CF patients in Bangladesh (151).

Non-coding RNA (ncRNA) such as microRNA (miRNA) has been implicated in many human diseases like CF, but little is known about their potential in therapeutics and CF research (158, 160). miRNA are short RNA molecules of about 22 nucleotides that regulate mRNA post-transcriptionally. One miRNA, miRNA-199a-5p, is even involved in the unfolded protein response (UPR) in chronic obstructive pulmonary disease and could prove to be an interesting target for CF research (159). In short, ncRNA like miRNA could be important to future CF research and the production of novel treatments.

To examine the effects that *P. aeruginosa* and arsenic have on CF and their relation to immunity and the regulation of ncRNA, a large RNA-Sequencing (RNA-Seq) experiment was designed (Figure 2.1). Zebrafish were injected with either 2.5 ng of a co-injection mixture of two CFTR MO or 2.5 ng of control MO at the 1-cell stage, as explained by Phennicie et al. (177). Starting after the initial collection of zebrafish embryos, arsenic was added to the egg water at 0, 2, or 10 parts per billion (ppb) and changed daily until RNA isolation. At 48 hours post fertilization (hpf) the zebrafish were injected with 50 CFU/embryo of *P. aeruginosa* into the Duct of Cuvier (DC). At 6 hours post infection (hpi) the zebrafish were homogenized and RNA was isolated for RNA-Seq. Both polyA+ selected mRNA and small RNA sequencing libraries were prepared for each sample and then sequenced allowing for characterization of mature mRNAs and small RNAs, such as miRNAs

Through analysis of the small RNA-Seq data, miRNA-199-1-3p (miRNA-199) was shown to be differentially expressed during infection and arsenic exposure (Figure 2.2.). miRNA-199 is highly conserved in both humans and zebrafish and has been associated with many potentially target protein-coding genes (190, 191). Several

experiments were conducted by Gagne and Sullivan et al. (unpublished) to understand the role miRNA-199 in innate immunity. The found miRNA-199 expression to increase by 2.4 fold upon CFTR KD and 10 ppb arsenic exposure at 6 hpi as measured by the RNA-Seq analysis (Figure 2.3a.). When targeted by a MO, miRNA-199 morphants displayed a significant reduction in bacterial burden at 6 hpi (Figure 2.3b). Furthermore, miRNA-199 KD markedly increased survival of zebrafish when infected with *P. aeruginosa* over a 5-day period, compared to control embryos infected with *P. aeruginosa* (Figure 2.3c) (Gagne and Sullivan et al., unpublished).

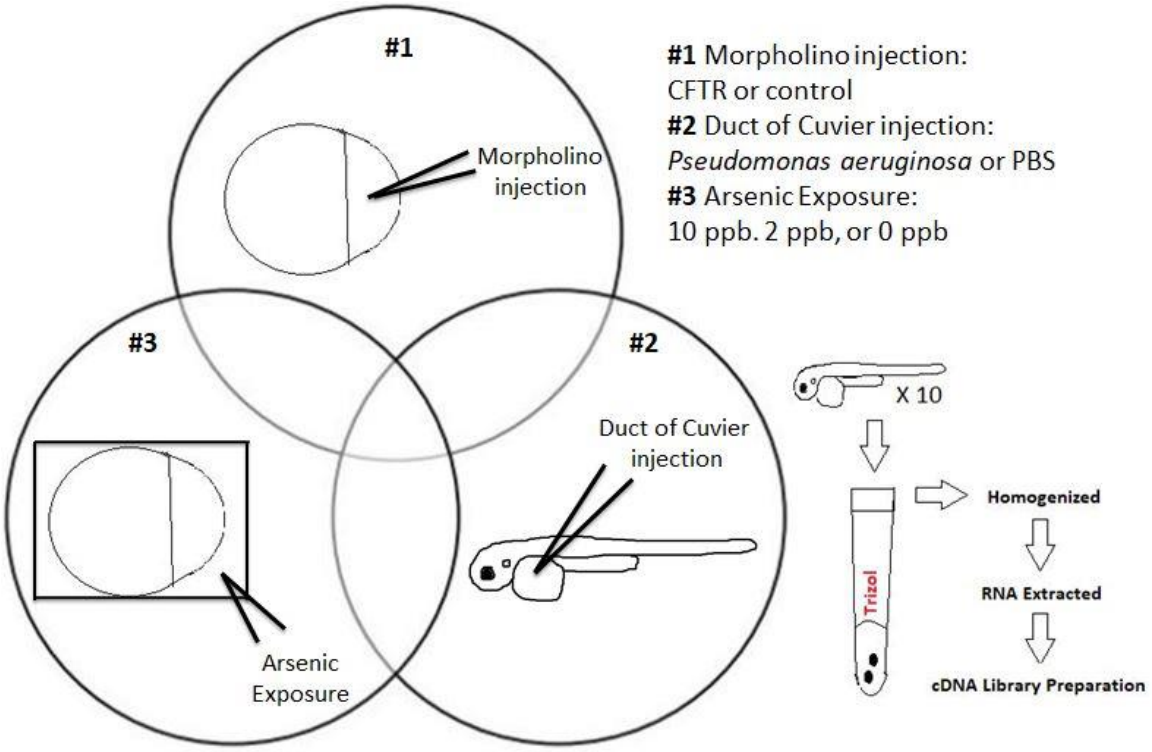


Figure 2.1. Experimental Design of RNA-Seq. Zebrafish embryos at the 1-cell stage were injected with 3 nl of a solution containing 0.05% phenol red, 1x Danieau buffer, nuclease-free water, and either 2.5 ng of a co-injection mixture of two CFTR MO or 2.5 ng of control MO as described by Phennicie et al. (177). Starting after the initial collection of zebrafish embryos and changed daily, arsenic was added to the egg water at 0, 2, or 10 parts per billion (ppb). At 48 hours post fertilization (hpf) the zebrafish were injected with 50 CFU/embryo of *P. aeruginosa* into the Duct of Cuvier (DC). At 6 hours post infection (hpi) the zebrafish were homogenized and RNA was isolated for cDNA library preparation and eventual RNA-sequencing.

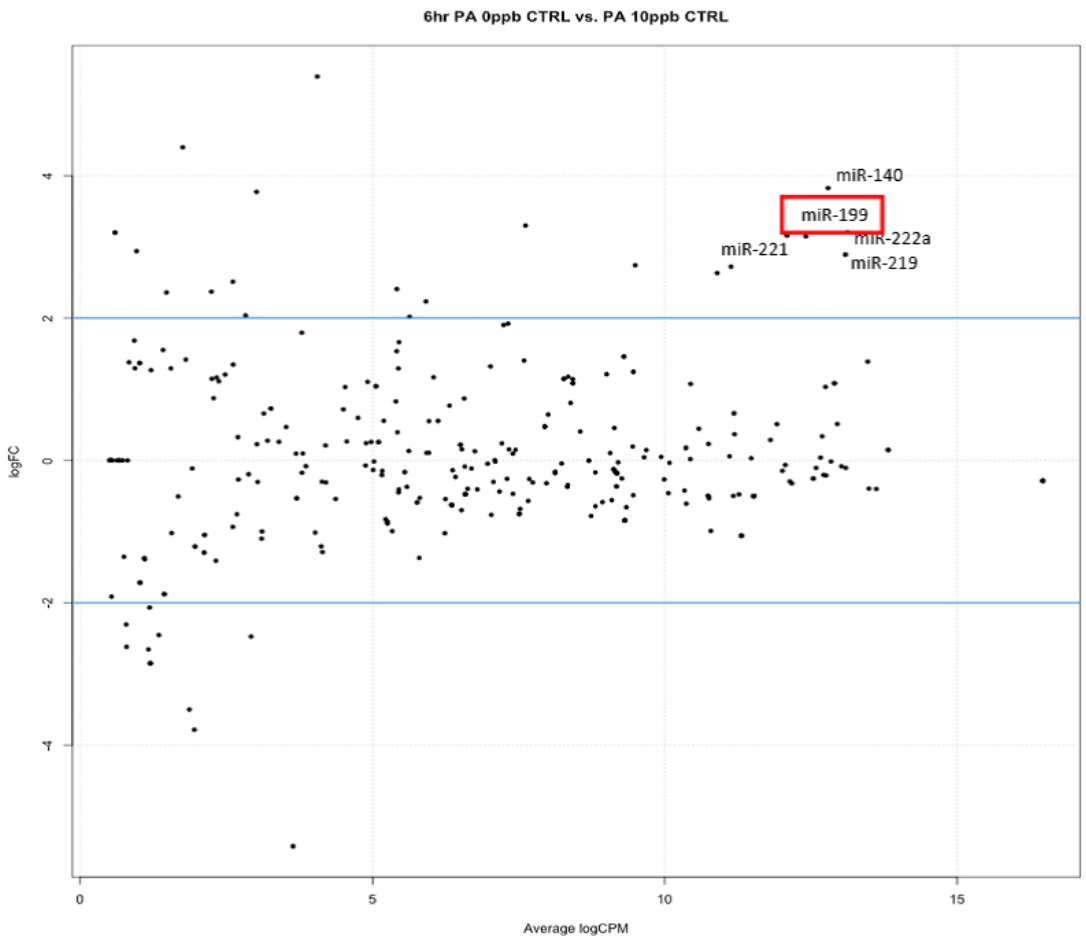


Figure 2.2. MA plot of miRNA-199 dysregulation. The MA plot compares the regulation of miRNA-199 during *P. aeruginosa* infection and 10 ppb arsenic exposure compared to control fish injected with PBS and not exposed to arsenic. miRNA-199 (miR-199) is more highly expressed and displayed an increased fold change compared to most other miRNAs assayed leading Gagne and Sullivan et al to investigate its function further. The y-axis represents $\log_2$ fold change and the x-axis represents the mean expression level ($\log_2$(Counts per million) units). Adapted from Gagne and Sullivan et al., unpublished data.

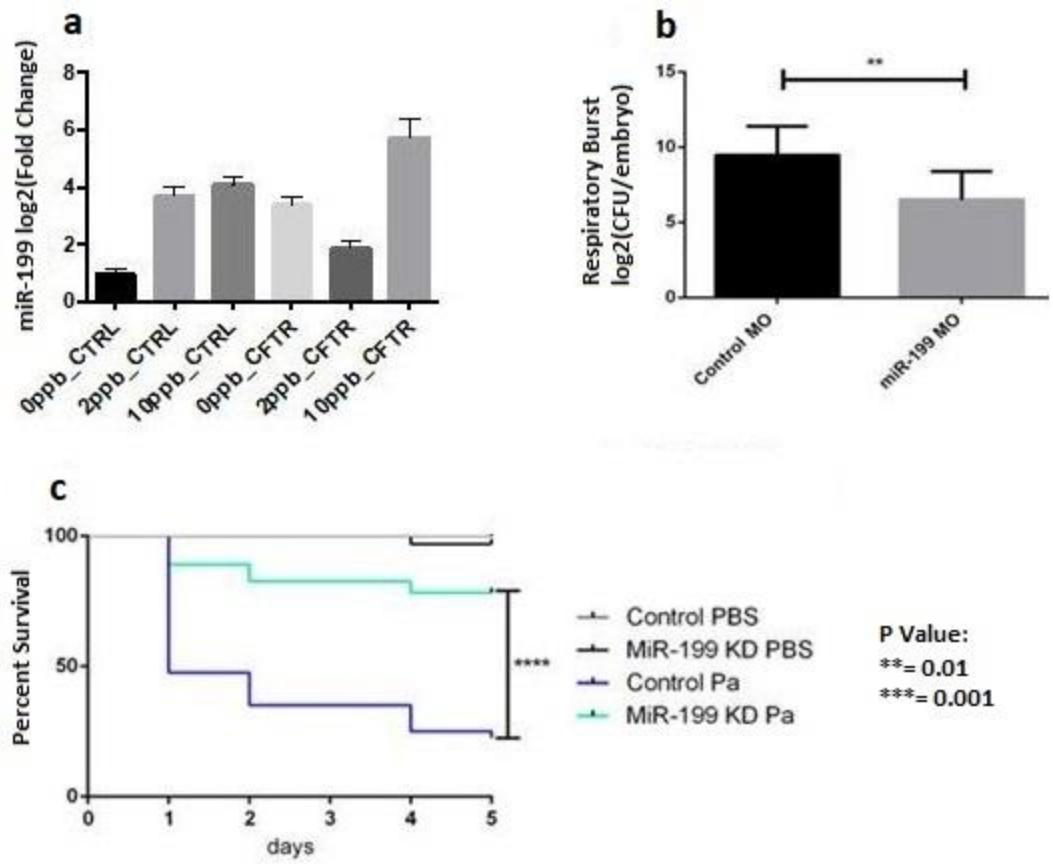


Figure 2.3. Knockdown of miRNA-199 improves response to infection by *P. aeruginosa.* (a) RNA-Seq reveals that miRNA-199 is differentially expressed in response to arsenic exposure in *cftr* morphants. (b) The respiratory burst response is significantly dampened in response miRNA-199 KD zebrafish embryos. (c) miRNA-199 KD leads to an increase survival in zebrafish embryos infection *P. aeruginosa* compared to zebrafish embryos injected with PBS. All experiments are representative of 3 biological replicates. Statistical analyses were calculated with the unpaired t test. Error bars represent standard error of the mean. Adapted from Gagne and Sullivan et al., unpublished data.

To further understand the protective role of miRNA-199 in innate immunity a bacterial burden and respiratory burst assay were conducted in the context of CFTR KD, arsenic exposure, and bacterial infection. The data from the bacterial burden was inconclusive due to a high degree of variability within each experiment. The respiratory burst, however, confirmed that miRNA-199 is having a protective role on ROS production in control zebrafish, which is consistent with Gagne and Sullivan et al. These results show that miRNA-199 is important in the modulation of the respiratory burst response during bacterial infection. In addition, these data further provide support for the research of ncRNA and their role in innate immunity and infection.

2.2. Materials and methods

2.2.1. Zebrafish care and maintenance

The zebrafish were maintained in agreement with the guidelines established by the University of Maine Institutional Animal Care and Use Committee (IACUC). Zebrafish embryos were collected at the one-cell stage of development from natural spawns of adult AB zebrafish. They were kept at 28℃ on a 16 hour light and 8 hour dark cycle. The zebrafish were grown in egg water (60 mg/L Instant Ocean sea salts), which was changed daily. Deceased zebrafish were collected with a plastic pipette and disposed of daily. Unused zebrafish, along with all fish at the end of each experiment, were euthanized by immersion in a lethal dose of tricaine followed by proper disposal.

2.2.2. Morpholino preparation and injection

Morpholinos (MO) were prepared as instructed by Gene Tools (Philomath, OR). CFTR morpholinos were generated against the zebrafish *cftr* gene (GenBank accession no. NM_001044883) as described by Phennicie et al. (177). Zebrafish *cftr* KD was achieved by injection a combination of CFTR I1E2 MO (5'-CCACCTGTAAATATTCAGAGCAGAT-3') and CFTR Trans MO (5'-CATCCTCCACAGGTGATCTCTGCAT-3'). CFTR I1E2 MO anneals to the boundary between intron 1 and exon 2 of the *cftr* transcript and yields a deletion of exon 2. CFTR Trans MO blocks translation of the *cftr* transcript by binding to its AUG start site. Embryos were co-injected with 1.25 ng of each CFTR MO.

miRNA-199 MO (Gene Tools, Philomath, OR) was designed using sequence information from the Sanger Institute's miRBase (Accession no. MIMAT0003155). miR-199-1-3p MO was designed to target the dicer cleavage site on the originally annotated mature (star) strand to prevent mature miRNA formation (5'-TCTAACCAATGTGCAGACTACTGTA-3'). Embryos were injected with 12 ng of MiR-199-1-3p MO into the yolk sac at the 1-2 cell stage.

Control MO (Gene Tools, Philomath, OR) was also injected into the 1-2 cell stage of zebrafish embryos (5'-CCTCTTACCTCAGTTACAATTTATA-3'). Control MO targets a human beta-globin intron mutation that causes beta-thalassemia. This MO, however, does not cause a change in phenotype and is used broadly as a negative control. Embryos were injected with 2.5 ng, 12 ng, or 14.5 ng control MO into the yolk sac. All MO injections were performed using a MPPI-2 microinjection apparatus (Applied

Scientific Instruments, Eugene, OR) using pulled microcapillary needles (Sutter Instruments, Novato, CA). After injection the zebrafish embryos were placed in 50 mL of egg water and maintained at 28℃.

2.2.3. Bacterial preparation

P. aeruginosa strain PA14 (p67T1) was used for all infections (Singer et al., 2010). PA14 (p67T1) contains a constitutive plasmid-encoded RFP variant of d-Tomato, which was engineered by Shaner et al. (192). Bacterial colonies were grown on 15 g/L bacteriological agar (Affymetrix, Cleveland, OH) combined with 25 g/L LB (Affymetrix, Cleveland, OH) supplemented with 750 µg of ampicillin/mL (Sigma-Aldrich, St. Louis, MO). Two days prior to infection, bacteria were streaked out on the aforementioned plates and grown for 24 hours at 37℃. The night before infection a single colony was picked using a p200 micropipettor tip and placed in a glass test tube containing 4 mL of 25 g/L LB broth (Affymetrix, Cleveland, OH) supplemented with 750 µg of ampicillin/mL (Sigma-Aldrich, St. Louis, MO). The test tube was placed in a shaking incubator (250 rpm) for 12-16 hours at 37℃. The following morning a sample of the bacterial culture was diluted 1:20 in a 1 mL cuvette with PBS (Life Technologies, Grand Island, NY) and measured using a spectrophotometer at 600 nm. An OD of 1 was added to 25 mL of 25 g/L LB (Affymetrix, Cleveland, OH) supplemented with 750 µg of ampicillin/mL (Sigma-Aldrich, St. Louis, MO) in a 125 mL Erlenmeyer flask and placed on a shaking incubator (250 rpm) for 2-4 hours. The culture was then added to a 50 mL plastic conical tube and centrifuged at 5000 x g for 5 min at 4℃. The supernatant was decanted and the pellet was re-suspended and washed in 3 ml of PBS (Life Technologies, Grand Island, NY). This step was repeated 3 times and the pellet re-suspended in 2 mL of

PBS (Life Technologies, Grand Island, NY). The culture was then decanted into a 5 mL syringe (BD, Franklin Lakes, NJ) connected to a 30 gauge x 1/2 needle (0.3 mm x 13 mm) (BD, Franklin Lakes, NJ) and expelled into a sterile 15 mL conical tube. This step was repeated twice and 50 µL of the bacterial culture was diluted 20x into 950 µL of PBS (Life Technologies, Grand Island, NY) in a clear plastic cuvette. The cuvette was read at 600 nm in a spectrophotometer. Bacterial cultures were diluted in PBS (Life Technologies, Grand Island, NY) and 50 µL of 5% phenol red (PR) (Sigma-Aldrich, St. Louis, MO) to achieve a 50 CFU/embryo injection. PR was used to visualize the injection into the embryo.

2.2.4. Bacterial injection

The zebrafish were dechorionated manually using Style 5 Dumont tweezers (Electron Microscopy Sciences, Hatfield, PA) at 2 days post fertilization (2dpf). Embryos were then anesthetized in 4 mg/mL Tricaine solution (Western Chemical, Ferndale, WA) and lined up on a 3% agar gel petri dish. *P. aeruginosa* was then injected into the embryo's Duct of Cuvier with 3 nL of *P. aeruginosa* strain PA14 (p67T1) to a final concentration of 50 CFU/embryo. The LD_{50} of PA14 (p67T1) was determined previously to be 50 CFU/embryo. This concentration resulted in 50% mortality by 48 hpi (Phennicie et al., 2010). Injection was done using a MPPI-2 microinjection apparatus (Applied Scientific Instruments, Eugene, OR) using pulled microcapillary needles (Sutter Instruments, Novato, CA). All embryos were screened with a Zeiss fluorescence screening microscope (Carl Zeiss AG, Oberkochen, Germany) for Texas Red fluorescence. Any embryos that were improperly injected and did not fluoresce red were removed and euthanized according to IACUC protocol with a lethal dose of Tricaine

(Western Chemical, Ferndale, WA). After injection, the zebrafish embryos were maintained at 28℃.

2.2.5. Arsenic exposure

A 10mM sodium arsenite ($NaAsO_2$) (Fisher Scientific, Hampton, NH) stock was made in 50 mL nanopure water and filtered with a 0.2 µM syringe filter. The filtered solution was stored at -20℃ in 1 mL aliquots in 1.5 mL microcentrifuge tubes. After MO injections zebrafish embryos were placed in 50 mL of egg water containing either 0 or 10 ppb arsenic. The egg water containing arsenic was changed daily.

2.2.6. Bacterial burden

At 8 hours post injection 20 zebrafish embryos from each treatment group (Table 2.1.) were washed in PBS (Life Technologies, Grand Island, NY). They were then individually added with 200 µL of PBS via a shortened p1000 tip on a p1000 micropipettor to separate 1.5 mL microcentrifuge tubes containing two 3.5 mm stainless steel balls (Next Advance, Averille Park, NY). The tubes were added to a Bullet Blender (Next Advance, Averille Park, NY) for 2 min at maximum speed and then centrifuged for 30 seconds at 13.5 x g in a desktop centrifuge. 100 µL from each tube was added to a separate agar plate containing 15 g/L bacteriological agar (Affymetrix, Cleveland, OH) combined with 25 g/L LB (Affymetrix, Cleveland, OH) supplemented with 750 µg of ampicillin/mL (Sigma-Aldrich, St. Louis, MO). Each sample was plated with a plastic, disposable culture stick and then placed in a 37℃ culture incubator for 24 hours. The CFU were counted and recorded after 24 hours. Error bars represent the standard error of

the mean (SEM). The data represent three independent trials were analyzed via ANOVA 4-factor analysis using JMP 8.01 (SAS, Cary, NC).

Table 2.1. Treatment groups for bacterial burden

Group No.	Arsenic Concentration (parts per billion)	2.5 ng *cftr* MO or 2.5 ng control MO (*cftr* or control)	12 ng miRNA-199 MO or 12 ng control MO (control or miRNA-199)	*P. aeruginosa* Infection (+ or -)
1	0 ppb	control	control	-
2	0 ppb	control	control	+
3	0 ppb	*cftr*	control	-
4	0 ppb	*cftr*	control	+
5	10 ppb	control	control	-
6	10 ppb	control	control	+
7	10 ppb	*cftr*	control	-
8	10 ppb	*cftr*	control	+
9	0 ppb	control	miRNA-199	-
10	0 ppb	control	miRNA-199	+
11	0 ppb	*cftr*	miRNA-199	-
12	0 ppb	*cftr*	miRNA-199	+
13	10 ppb	control	miRNA-199	-
14	10 ppb	control	miRNA-199	+
15	10 ppb	*cftr*	miRNA-199	-
16	10 ppb	*cftr*	miRNA-199	+

2.2.7. Respiratory burst assay

The respiratory burst assay was performed as described by Hermann et al. and Goody et al. (187, 188). The treatment groups are categorized in Table 2.2. Twelve embryos per treatment group were exposed to phorbol 12-myristate 13-acetate (PMA) and 12 were not. The plate was read using a Synergy2 plate reader (Biotek, Winooski, VT). The plate was shaken for 5 seconds and then measurements were taken with an excitation of 485 nm and emission of 528 nm. Error bars represent SEM. The data

represent three independent trials and were analyzed via ANOVA 4-factor analysis using JMP 8.01 (SAS, Cary, NC).

Table 2.2. Treatment groups for respiratory burst assay

Group No.	Arsenic Concentration (parts per billion)	2.5 ng *cftr* MO or 2.5 ng control MO (*cftr* or control)	12 ng miRNA-199 MO or 12 ng control MO (control or miRNA-199)	PMA (+ or -)
1	0 ppb	control	control	-
2	0 ppb	control	control	+
3	0 ppb	*cftr*	control	-
4	0 ppb	*cftr*	control	+
5	10 ppb	control	control	-
6	10 ppb	control	control	+
7	10 ppb	*cftr*	control	-
8	10 ppb	*cftr*	control	+
9	0 ppb	control	miRNA-199	-
10	0 ppb	control	miRNA-199	+
11	0 ppb	*cftr*	miRNA-199	-
12	0 ppb	*cftr*	miRNA-199	+
13	10 ppb	control	miRNA-199	-
14	10 ppb	control	miRNA-199	+
15	10 ppb	*cftr*	miRNA-199	-
16	10 ppb	*cftr*	miRNA-199	+

2.3. Results

2.3.1. miRNA-199 shows inconclusive effect on overall *P. aeruginosa* bacterial burden

The preliminary experiments from Gagne and Sullivan et al. have revealed a role for miRNA-199 in the respiratory burst response to bacterial infection in zebrafish. A bacterial burden was therefore conducted to determine if miRNA-199 was effecting the overall bacterial concentration in the zebrafish. Zebrafish were grown up to 48 hpf and

injected with 50 CFU/embryo *P. aeruginosa* or PBS. At 8 hpi the zebrafish were homogenized and plated separately. The plates were counted 24 hours later, but did not reveal conclusive results (Figure 2.4.). The three biological replicates that were performed were inconsistent. Replicate 1 (Figure A.1.) and replicate 2 (Figure A.2.) showed opposite effects for zebrafish co-injected with control and miRNA-199 MOs compared to control zebrafish. Replicate 2 showed miRNA-199 KD causing a decrease in bacterial burden where replicate 1 showed an increase bacterial burden. Replicate 2 showed an increase in bacterial burden when zebrafish were exposed to 10 ppb arsenic compared to 0 ppb arsenic. In addition, CFTR KD appeared to reduce bacterial burden when compared to control. miRNA-199 KD in CFTR KD zebrafish showed an increased burden when compared to CFTR KD control fish. Furthermore, replicate 3 (Figure A.3.) showed no significant change in the data.

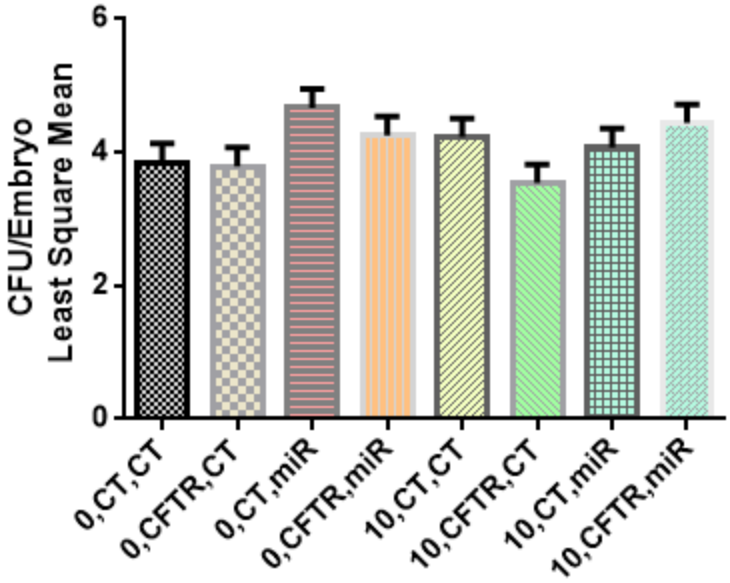


Figure 2.4. miRNA-199 shows inconsistent effects on *P. aeruginosa* bacterial burden. Three biological replicates were combined and analyzed using a four-factor ANOVA statistical analysis. The results show no significant change between the experimental groups and when the separate replicates were analyzed for statistical significance there appeared to be no discernable trend within the experimental groups between the trials. The biological replicates are shown individually in Appendix A.

2.3.2. miRNA-199 plays a role in the regulation of respiratory burst

A respiratory burst assay (RBA) was performed to elucidate the role of miRNA-199 in zebrafish that were co-injected with CFTR and exposed to arsenic. Our controls show that when CFTR MO is co-injected with control MO a reduced respiratory burst is observed. In addition, arsenic decreased the respiratory burst. When zebrafish are co-injected with control and miRNA-199 MOs and compared to control zebrafish they

display a dampened production of reactive oxygen species (ROS). In addition, in the context of arsenic, CFTR KD causes a decrease in ROS production when compared to control. Furthermore, in zebrafish that were co-injected with CFTR and miRNA MOs an increased respiratory burst was observed when compared to both zebrafish that were co-injected with CFTR and control MOs and zebrafish that were injected with control and miRNA-199 MOs.

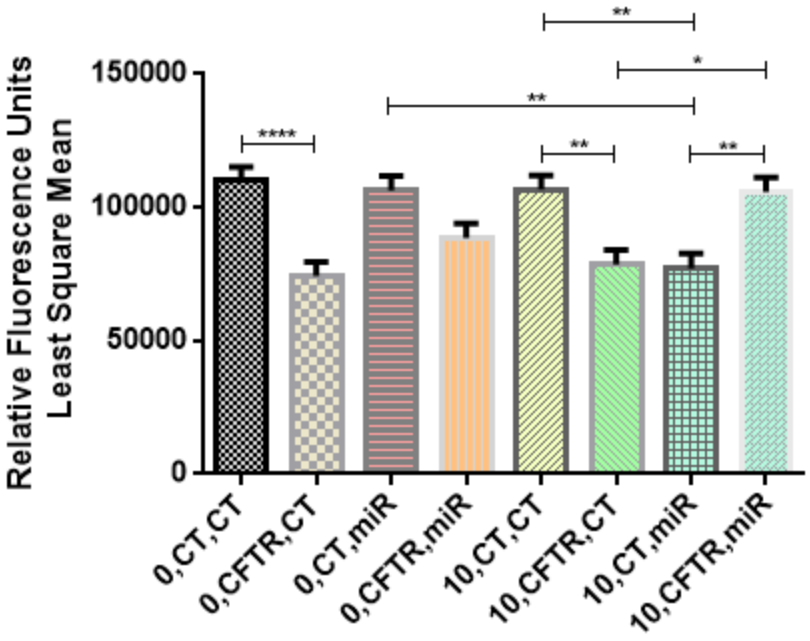


Figure 2.5. miRNA-199 plays a role in the regulation of respiratory burst. Three biological replicates of respiratory burst assays were combined and analyzed with a 3-factor ANOVA statistical analysis. Regardless of arsenic exposure CFTR KD leads to a dampened respiratory burst. Arsenic exposure also dampens ROS production in zebrafish co-injected with control and miRNA-199 MOs. Zebrafish that were exposed to arsenic and co-injected with control and miRNA-199 MOs also showed dampened ROS production when compared to control zebrafish exposed to arsenic. Conversely, when zebrafish co-injected with CFTR and miRNA-199 MOs were compared to the zebrafish co-injected with control and miRNA-199 MOs an increased respiratory burst was observed in the zebrafish co-injected with CFTR and miRNA-199 MOs.

2.4. Discussion

Unpublished data by Gagne and Sullivan et al. showed a protective role for miRNA-199 in immunity and infection. The results showed that miRNA-199 KD resulted in a dampened respiratory burst response in wild-type zebrafish along with increased survival in miRNA-199 KD fish compared to control KD fish infected with *P. aeruginosa*. At first glance these two results appear to be conflicting, but it is possible that reducing the respiratory burst is increasing survival by limiting collateral damage from PMN respiratory burst. An immune response that is too intense leads to damage, while an immune response that is too weak will result in decreased pathogen clearance and yield higher bacteria burden. In CF patients PMNs and their markers are positively correlated with decreased lung function (44). CF patients are unable to clear pathogens due to the ineffectiveness of PMNs and the virulence factors of *P. aeruginosa.* These individuals are at risk for unresolved infections as a result of chronic inflammation (112, 193). These results together suggest that miRNA-199 is an important mediator of immunity and possibly imply a role in regulation of ROS which can lead to a more controlled immune response during infection. In addition, miRNA-199 KD could potentially rescue respiratory burst and bacterial burden in CFTR KD zebrafish.

To determine if the protective role of miRNA-199 translated to decreased bacterial burden, which is suggested by its role in increased survival, a bacterial burden assay was conducted. Three biological replicates were performed, with zebrafish that were injected with either CFTR or control MOs and either miRNA-199 or control MOs, exposed to 10 or 0 ppb arsenic, and infected with *P. aeruginosa* or control (PBS). When the three replicates were compiled and a 4-factor ANOVA statistical analysis was

performed, no significant change between the experimental groups was observed. The three biological replicates were inconsistent with each other and therefore no conclusions could be drawn about the role of miRNA-199 in infection. We expected arsenic and CFTR KD to increase the bacterial burden as described by Nayak et al, and Phennicie et al, respectively (147, 177). In addition, for the same reasons outlined above, we expected miRNA-199 KD to decrease bacterial burden and correlate with the increased survival observed by Gagne and Sullivan et al., but our results were too inconsistent to permit us to reach any conclusion.

Although the replicates are not consistent with each other, replicate 2 (A.2.) does reveal a trend that is consistent with previously published data (147, 177). Arsenic was shown to increase the bacterial burden in zebrafish when compared to zebrafish not exposed to arsenic. In addition, miRNA-199 and control co-injected zebrafish displayed decreased bacterial burden when compared to control zebrafish. Conversely, zebrafish co-injected with CFTR and miRNA-199 MOs resulted in an increased burden when compared to fish co-injected with CFTR and control MOs. These results appear to be in disagreement and suggest a role for miRNA-199 in CFTR KD zebrafish, but to fully elucidate this role more studies must be conducted.

In addition to the bacterial burden experiments, a respiratory burst assay was conducted to understand the role of miRNA-199 during infection and arsenic exposure in CFTR KD zebrafish. As expected, we saw that arsenic exposure decreased the overall respiratory burst and CFTR KD also decreased respiratory burst as previously described (147, 177). Similar to the results shown in Figure A.2., Figure 2.5. shows that zebrafish co-injected with miRNA-199 and control MO had a dampened ROS production when

compared to control zebrafish. This result confirms the results of Gagne and Sullivan et al. Conversely, in the context of CFTR the opposite results are true. In zebrafish co-injected with CFTR and control MOs and zebrafish co-injected with control and miRNA-199 MOs both had dampened respiratory bursts when compared to zebrafish co-injected with CFTR and miRNA-199 MOs. This result is also shown in Figure A.2. and was not an expected outcome. In fact, there are many current strategies being studied to modulate the PMN-mediated inflammation, which has been correlated with decreased lung function (112, 194-197). Modulation of inflammation via miRNA-199 KD in our experiments, in which it was expected that ROS production would decrease, actually led to an increase in ROS production and a trend toward increased bacterial burden. Therefore, there appears to be a novel role for CFTR and miRNA-199 KD that leads to both an increased bacterial burden and respiratory burst. CF patients from an early age had decreased iNOS expression, which has been implicated in reduced bacterial clearance (27, 116, 117, 119). Potentially, the further decrease in NO^- could be leading to an increased bacterial burden, which in turn is leading to an increased immune response and subsequently an increase in ROS.

In conclusion, a potential role for miRNA-199 in regulating the production of ROS and aiding in the reduction of the bacterial burden in control fish has been established. Although, the data from the bacterial burden experiments were not significant, a trend is suggested that is consistent with the RBA data, pointing to a potential role for miRNA-199 in protecting zebrafish from infection by innate immune regulation. More biological replicates of the bacterial burden assay would be necessary before any conclusions about a role miRNA-199 might have in overall bacterial

concentration could be made, but it is clear that miRNA-199 has a significant role in the production of ROS. In addition, it has become apparent that zebrafish co-injected with CFTR and miRNA-199 MOs display increased burden and ROS production, which was not an expected result. Additional research is necessary to understand why miRNA-199 in conjunction with CFTR KD is increasing respiratory burst and potentially increasing bacterial burden.

Future experiments must include repeating the bacterial burden experiment to account for the high degree of variability found within the three biological replicates. In addition, overexpression of miRNA-199 could be performed, followed by a survival study, bacterial burden, and respiratory burst assay. If during overexpression of miRNA-199 decreased bacterial burden and/or respiratory burst was observed in CFTR KD zebrafish it will help to both validate our current results and aid us in understanding why miRNA-199/CFTR KD zebrafish display an increased bacterial burden and respiratory burst.

miRNA-199 is a highly conserved miRNA in zebrafish and humans and its targets have been shown to be involved in regulation of the metabolic process, regulation of signaling, cell communication, signal transduction, and response to stimulus (190, 191, 198). This suggests that miRNA-199 is involved in many area of biology and in future experiments its targets could be knocked-down in zebrafish to understand their relationship to miRNA-199. In addition, following a KD or overexpression of miRNA-199 a RNA-Seq experiment could be conducted and the dysregulation of protein-coding genes along with ncRNA could be analyzed. These experiments could help to further

elucidate targets of miRNA-199 and increase our understanding of its role in immunity and infection.

CHAPTER 3

CANDIDATE LINCRNAs VALIDATE RNA-SEQ VIA RT-QPCR AND PLAY A ROLE ININFECTION BY *P. AERUGINOSA*

3.1. Introduction

Cystic fibrosis (CF) is a lethal hereditary disease that is characterized by a mutation in both copies of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a chloride ion channel present in most epithelial cells and a variety of other cell types. The most common form of this disease is the deletion of a phenylalanine amino acid at position 508 termed ΔF508, which accounts for roughly 70% of cases worldwide and 90% of the cases in the United States. Although ΔF508 mutations make up the vast majority of CF cases, there are greater than 2000 other mutations that cause the disease known as CF (1, 199). These mutations lead to a multitude of different problems with the CFTR protein, but the end result is almost always the same, thick mucus formation. The thick, sticky mucus is formed as a result of improper ion trafficking due to the mutated chloride ion channel, which causes an assortment of issues in the gastrointestinal, endocrine, reproductive, and respiratory systems. Although CF is a multi-organ disorder, it is usually referred to as a lung disease because the overwhelming majority (>90%) of CF patients ultimately die from persistent lung infection (1, 23).

The most prevalent microorganism in adults with CF is *Pseudomonas aeruginosa*, a gram-negative, opportunistic, and ubiquitous pathogen that employs an arsenal of devastating virulence factors. Among these virulence factors is the quorum sensing (QS) system that is crucial in the production of virulence factors through multicomponent

communication and regulatory networks. The QS system is responsible for the production of biofilms, which give *P. aeruginosa* increased resistance to opsonization, destruction, and antibiotic therapies (41, 42, 46, 47). *P. aeruginosa* is also known to adapt to host pressures and evolve to a mucoid form, which is responsible for the chronic lung infections and is largely blamed for its lethality (53).

In addition to *P. aeruginosa*, environmental factors such as arsenic have been recently studied as a confounding factor to the health of individuals living with CF. Arsenic has been shown to reduce the respiratory burst of immune cells in response to viral and bacterial infection in zebrafish (*Danio rerio*) while at the same time increasing the release of antiviral and antibacterial cytokines (147). Interestingly, arsenic has also been connected to c-Cbl-mediated ubiquitination and degradation of CFTR in the human lung (149).). Furthermore, arsenic concentration in well water in Bangladesh has been correlated with higher than normal sweat conductivity in non-CF patients (151). Arsenic is a complicating factor in the already difficult CF puzzle.

Non-coding RNA (ncRNA), such as long intergenic non-coding RNAs (lincRNA), have been implicated in CF, but little is known about their potential in therapeutics and CF research (152). lincRNAs are a member of the long non-coding RNA (lncRNA) family that are characterized by not being included in any part of other genes. lincRNAs function via a variety of proposed mechanisms including cotranscriptional regulation, regulation of gene expression, acting as molecular decoys, and acting as cytoplasmic scaffolds (153). Research has been conducted to catalog lincRNA loci and transcripts across a multitude of species including the zebrafish. Interestingly, lincRNA have been shown to be differentially expressed between CF and non-CF individuals with

many of the differentiated lincRNAs being related to inflammation in CF bronchial epithelium cells (152). It can be concluded that studies of lincRNA and its relationship with CF could give rise to novel therapies for CF.

In a search for ways to study the effect that these biological factors have on individuals with CF *in vivo*, the zebrafish is an attractive, viable test system. The zebrafish is an optically clear member of the teleost family of vertebrates that has become a powerful tool in the study of diseases and immunity due to its ease of breeding and infection, high fecundity, and relatively low cost. In addition, the zebrafish is genomically and physiologically similar to humans and depends solely on an innate immune system for the first 4-6 weeks of life (172, 174, 175). Importantly, the zebrafish is an excellent model for the study CF and *P. aeruginosa* infection (177). During CFTR knockdown (KD) using a morpholino oligonucleotide (MO) the zebrafish displayed a dampened respiratory burst and decreased bacterial burden (177).

RNA-Sequencing analysis elucidated the expression profile of ncRNA by environmental interactions in innate immunity (Figure 2.1.). Zebrafish were injected with MO at the 1-cell stage with either 2.5 ng of a co-injection mixture of two CFTR MO or 2.5 ng of control MO. Arsenic was added to the egg water at a concentration of 0, 2, or 10 parts per billion (ppb) and changed daily with the egg water. At 48 hours post fertilization (hpf) the zebrafish were injected with 50 CFU/embryo of *P. aeruginosa* into the Duct of Cuvier (DC). At 6 hours post infection (hpi) the zebrafish were homogenized and RNA was isolated for RNA-Seq.

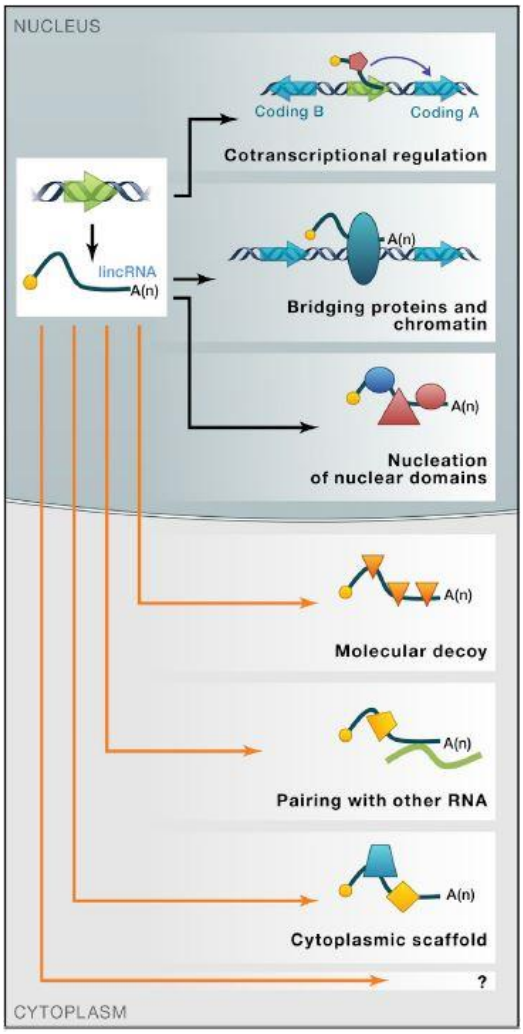


Figure 3.1. Proposed mechanisms for lincRNA function. lincRNAs are thought to function in many different ways including cotranscriptional regulation by either interacting with factors or transcribing through a regulatory region, the *cis* or *trans* regulation of gene expression, interaction with RNA-binding factors, or initiating posttranscriptional regulation via binding with other RNA transcripts. In addition, there are believed to be more functions that have yet to be discovered. Adapted from: Ulitsky I, Bartel DP. 2013. lincRNAs: genomics, evolution, and mechanisms. Cell 154:26-46.

The resulting RNA-Seq reads were aligned to lincRNAs annotated from three recent studies of zebrafish lincRNAs and their loci, and several candidate lincRNA were elucidated (161, 169, 170). The lincRNA of interest were differentially expressed when zebrafish were infected with *P. aeruginosa*. To validate the RNA-Seq data, an identical experiment was run to mirror the *P. aeruginosa* infection from the RNA-Seq experiment. Zebrafish were infected with *P. aeruginosa* at 48 hpf and RNA was collected at 3, 6, and 9 hpi. The RNA was then converted to cDNA and qPCR was performed. Initially a panel of inflammatory cytokines was profiled to confirm innate immune response to *P. aeruginosa*, which resulted in a strong, significant upregulation in all cytokines assayed across the majority of timepoints. Next, the expression of the candidate lincRNAs, all of which are not characterized in any species, was measured. Linc-mettl3 was found to be significantly upregulated at 3 hpi but not at 6 hpi, which did not corroborate the RNA-Seq data. Although linc-mettl3 did not validate the RNA-Seq data, linc-cyb5a and linc-zgc:113057_2 showed statistically significant trends that mirrored the RNA-Seq data. Interestingly, linc-zgc:113057_2 was found to have increased almost 34-fold in the qPCR analysis, which is an approximately 32 fold increase compared to the RNA-Seq data. Furthermore, it was found that linc-zgc:113057_2 has a neighboring protein coding gene, zgc:113057, which is differentially expressed at 3 and 9 hpi. These findings help to not only validate the RNA-Seq data, but also to our knowledge this is the first time that lincRNAs have been shown to be modulated during bacterial infection in zebrafish and among only a few in other animal models. There is no cure for CF and bacterial infections are still difficult to control, but these results inform new hypotheses for future experiments that could potentially produce novel CF treatments and bacterial therapies.

3.2. Materials and methods

3.2.1. Zebrafish care and maintenance

The zebrafish were maintained in agreement with the guidelines established by the University of Maine Institutional Animal Care and Use Committee (IACUC). Zebrafish embryos were collected at the one-cell stage of development from natural spawns of adult AB zebrafish. They were kept at 28℃ on a 16 hour light and 8 hour dark cycle. The zebrafish were grown in egg water (60 mg/L Instant Ocean sea salts), which was changed daily. Deceased zebrafish were collected with a plastic pipette and disposed of daily. Unused zebrafish, along with all fish at the end of each experiment, were euthanized by immersion in a lethal dose of tricaine followed by proper disposal.

3.2.2. Bacterial preparation

P. aeruginosa strain PA14 (p67T1) was used for all infections (Singer et al., 2010). PA14 (p67T1) contains a constitutive plasmid-encoded RFP variant of d-Tomato, which was engineered by (192). Bacterial colonies were grown on 15 g/L bacteriological agar (Affymetrix, Cleveland, OH) combined with 25 g/L LB (Affymetrix, Cleveland, OH) supplemented with 750 µg of ampicillin/mL (Sigma-Aldrich, St. Louis, MO). Two days prior to infection bacteria were streaked out on the aforementioned plates and grown for 24 hours at 37℃. The night before the infection experiment a single colony was picked using a p200 micropipettor tip and placed in a glass test tube containing 4 mL of 25 g/L LB broth (Affymetrix, Cleveland, OH) supplemented with 750 µg of ampicillin/mL (Sigma-Aldrich, St. Louis, MO). The test tube was placed in a shaking incubator (250 rpm) for 12-16 hours at 37℃. The following morning a sample of the

bacterial culture was diluted 1:20 in a 1 mL cuvette with PBS (Life Technologies, Grand Island, NY) and measured using a spectrometer at 600 nm. An OD of 1 was added to 25 mL of 25 g/L LB (Affymetrix, Cleveland, OH) supplemented with 750 µg of ampicillin/mL (Sigma-Aldrich, St. Louis, MO) in a 125 mL Erlenmeyer flask and placed on a shaking incubator (250 rpm) for 2-4 hours. The culture was then added to a 50 mL plastic conical tube and centrifuged at 5000 x g for 5 min at 4℃. The supernatant was decanted and the pellet was resuspended and washed in 3 ml of PBS (Life Technologies, Grand Island, NY). This step was repeated 3 times and was then resuspended in 2 mL of PBS (Life Technologies, Grand Island, NY). The culture was then decanted into a 5 mL syringe (BD, Franklin Lakes, NJ) connected to a 30 gauge x 1/2 needle (0.3 mm x 13 mm) (BD, Franklin Lakes, NJ) and expelled into a sterile 15 mL conical tube. This step was repeated twice. 50 µL of the bacterial culture was diluted 20x into 950 µL of PBS (Life Technologies, Grand Island, NY) into a clear plastic cuvette. The cuvette was read at 600 nm in a spectrophotometer. Bacterial cultures were diluted in PBS (Life Technologies, Grand Island, NY) and 50 µL of 5% phenol red (PR) (Sigma-Aldrich, St. Louis, MO) to achieve a 50 CFU/embryo injection. PR was used to visualize the injection into the embryo.

3.2.3. Bacterial injection

The zebrafish were dechorionated manually using style 5 Dumont tweezers (Electron Microscopy Sciences, Hatfield, PA) at 2 days post fertilization (2dpf). Embryos were then anesthetized in 4 mg/mL Tricaine solution (Western Chemical, Ferndale, WA) and lined up on a 3% agar gel petri dish. *P. aeruginosa* was then injected into the embryo's Duct of Cuvier with 3 nL of *P. aeruginosa* strain PA14 (p67T1) to a final

concentration of 50 CFU/embryo. The LD_{50} of PA14 (p67T1) was determined previously to be 50 CFU/embryo. This concentration resulted in 50% mortality by 48 hpi (177). Injection was done using a MPPI-2 microinjection apparatus (Applied Scientific Instruments, Eugene, OR) using pulled microcapillary needles (Sutter Instruments, Novato, CA). All embryos were screened with a Zeiss fluorescence screening microscope (Carl Zeiss AG, Oberkochen, Germany) for Texas Red fluorescence. Any embryos that were improperly injected and did not fluoresce red were removed and euthanized according to IACUC protocol with a lethal dose of Tricaine (Western Chemical, Ferndale, WA). Post injection, the zebrafish embryos were maintained at 28℃.

3.2.4. RT-qPCR primer design

Primers of candidate lincRNAs were designed using Integrated DNA Technologies (IDT) PrimerQuest Tool. Primers were diluted to 10 µM. The sequences are listed in Table 3.1.

Table 3.1. RT-qPCR lincRNA primers

Primer Name	Forward Sequence	Reverse Sequence
linc-mettl3	AGAAGATCCTCTGCGCTTTAC	CTTCACTCACTACAGCAGTCTC
linc-zgc:158317	AGTTCATGTTAGAGCAGGTACAA	GACTTGCAGCAACCAATACAC
zgc:113057_2	GGACTGTGCATTCTGGGTAATC	CCTTCTGATGCCTTTGCTTTG
linc-cyb5a	GACGAGTGACAGAGAACATCAA	CACAGACCAAACAAACCAAGAG
linc-loc555288	CATCGCCACTCAGACAATCA	TCCAGAACAACTTCACCTACAC
linc-zgc:113057_2	CATTCAGATCATCCTCAGATCCC	GTACTGGCTCACTGTCTTGTT

3.2.5. RT-qPCR

Total RNA was isolated from infected embryos at 3, 6, and 9 hours post infection (hpi). Ten fish per treatment were homogenized in a Bullet Blender (Next Advance, Averille Park, NY) in 200 µL Trizol (Thermo Fisher Scientific, Waltham, MA) in separate 1.5 mL microcentrifuge tubes containing two 3.5 mm stainless steel beads (Next Advance, Averille Park, NY). All treatment groups were prepared in triplicate. RNA was isolated using the Direct-Zol Miniprep Plus kit (Zymo Research, Irvine, CA). RNA was then converted into cDNA via the iScript Reverse Transcriptase Supermix for RT-qPCR kit (Bio Rad, Hercules, CA). The cDNA synthesis was performed with 500 µg of RNA sample per replicate. After the conclusion of the cDNA synthesis the samples were diluted with 180 µL of nuclease-free water. RT-qPCR was done utilizing PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD). RT-qPCR was performed with a CFX96 Real-Time System (Bio Rad, Hercules, CA). Error bars represent SEM. The data represent three independent trials and statistical significance was determined by an unpaired t test (GraphPad Prism 6, La Jolla, CA).

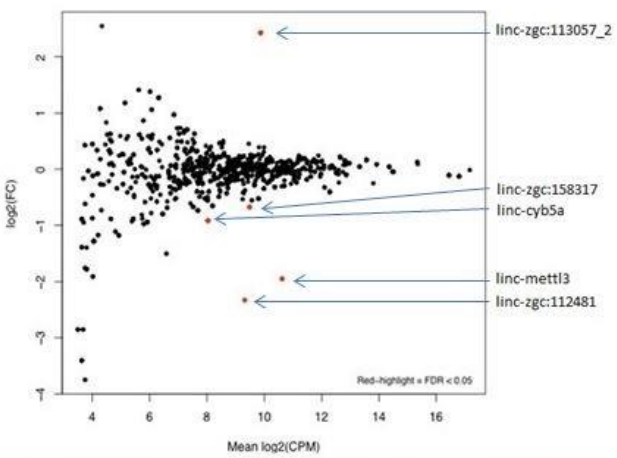
3.3. Results

3.3.1. Determination of candidate lincRNAs from RNA-Seq data

The expression of lincRNAs was examined by aligning RNA-Seq reads to catalogs of lincRNA loci (161, 169, 170). This present study focused on the differential expression of zebrafish lincRNAs in fish infected with *P. aeruginosa* alone, i.e. without *cftr* KD or arsenic exposure. This subset of the RNA-Seq was examined to elucidate lincRNAs that play a role in infection in order to find potential targets for improving *P.*

aeruginosa clearance. The loci elucidated from the three references listed were cross referenced with the RNA-Seq data, and a small subset of the lincRNAs showed significant dysregulation during *P. aeruginosa* infection, including: linc-mettl3, linc-zgc:113057_2, linc-zgc:158317, linc-cyb5a, and linc-loc555288 (Figure 3.2a.). These genes were chosen based on their strong $\log_2$ fold change and a P value and discovery rate (FDR) below the 0.05 threshold (Figure 3.2b.).

a



b

Linc-RNA	Log_2Fold Change	P Value	FDR
Linc-mettl3	-1.95	3.60E-14	1.95E-11
Linc-zgc:113057_2	2.43	9.50E-14	2.85E-11
Linc-zgc:158317	-3.33	0.0001	0.014
linc-cyb5a	-0.67	0.0003	0.034
Linc-zgc:112481	-0.65	0.0009	0.066

Figure 3.2. Five differentially expressed lincRNAs identified in zebrafish infected with 50 CFU/embryo at the 48 hpi. (a) A MA plot represents expression data of the lincRNAs found in Ulitsky et al. (169). Each dot represents a different lincRNA with the red dots representing lincRNAs that have a P value and FDR below 0.05. The plot displays the log_2 fold change on the y-axis and the mean expression on the x-axis. (b) The lincRNA represented above as red dots are listed with their log_2 fold change, P value, and FDR (false discovery rate). A P value and FDR below 0.05 is considered statistically significant.

3.3.2. Three candidate lincRNAs are found to be protein-coding genes and one is neighboring a lincRNA of interest.

After examining current gene annotations of two candidate lincRNAs using the University of California Santa Cruz (UCSC) Genome Browser (200), both linc-zgc:158317 and linc-loc555288 were found to be protein-coding genes and were therefore removed from further consideration. linc-zgc:113057_2 was also determined to be a protein-coding gene named zgc:113057, which has a neighboring lincRNA called linc-zgc:113057_2 (Figure 3.2). Therefore, linc-zgc:113057_2 was kept as a candidate lincRNA with the corrected loci. In addition, it was found that linc-zgc:113057_2 is very strongly upregulated during *P. aeruginosa* infection when compared to PBS injected zebrafish. zgc:113057_2 was not found to be significantly dysregulated during *P. aeruginosa* infection.

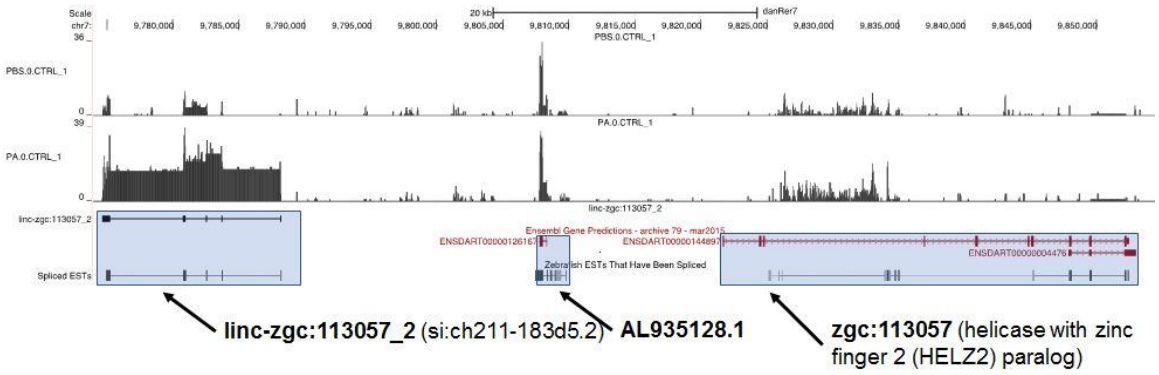


Figure 3.3. The coordinates for linc-zgc:113057_2 taken from Ulitsky et al. were determined to yield a protein-coding gene named zgc:113057 (2011). A neighboring lincRNA, linc-zgc:113057_2 was found relatively close to zgc:113057 on chromosome 7 and was subsequently added to the list of candidate lincRNAs. In addition, in the second expression profile that linc-zgc:113057_2 appears to be strongly differentially expressed during *P. aeruginosa* (labeled at PA above) infection when compared to the first row showing expression during a PBS injection (control). Zgc:113057 does not appear to be differentially regulated during *P. aeruginosa* infection.

3.3.3. Inflammatory cytokines are significantly and strongly upregulated during *P. aeruginosa* infection

After determination of candidate lincRNAs, zebrafish were infected with P. aeruginosa at 48 hpi to mirror the design of the RNA-seq bacterial injections. This was done to both validate the RNA-Seq expression data and to further characterize the candidate lincRNAs. To verify that the zebrafish were properly infected and displayed a characteristic innate immune response, a panel of inflammatory cytokines were profiled via RT-qPCR (Figure 3.4.). IL-1, Serum-amyloid A (SAA), CXCL8-L1, CXCL8-L2

(CXCL8-L1 and CXCL8-L2 are homologues in the zebrafish and are referred to as IL-8 in humans), and tumor necrosis factor α (TNFα) were found and a characteristic expression profile was observed. All 5 of the cytokines displayed strong and significant upregulation of expression at 3, 6, and 9 hpi with the exception of CXCL8-L1. CXCL8-L1did not have a significant change in expression at 9 hpi and, although still significant, it did not display a relatively large fold change at 6 hpi when compared to 3 hpi. The strong and significant upregulation of the inflammatory cytokines showed that a systemic bacterial infection was achieved. In addition, once the infection was confirmed, the expression of the candidate lincRNAs could be assayed.

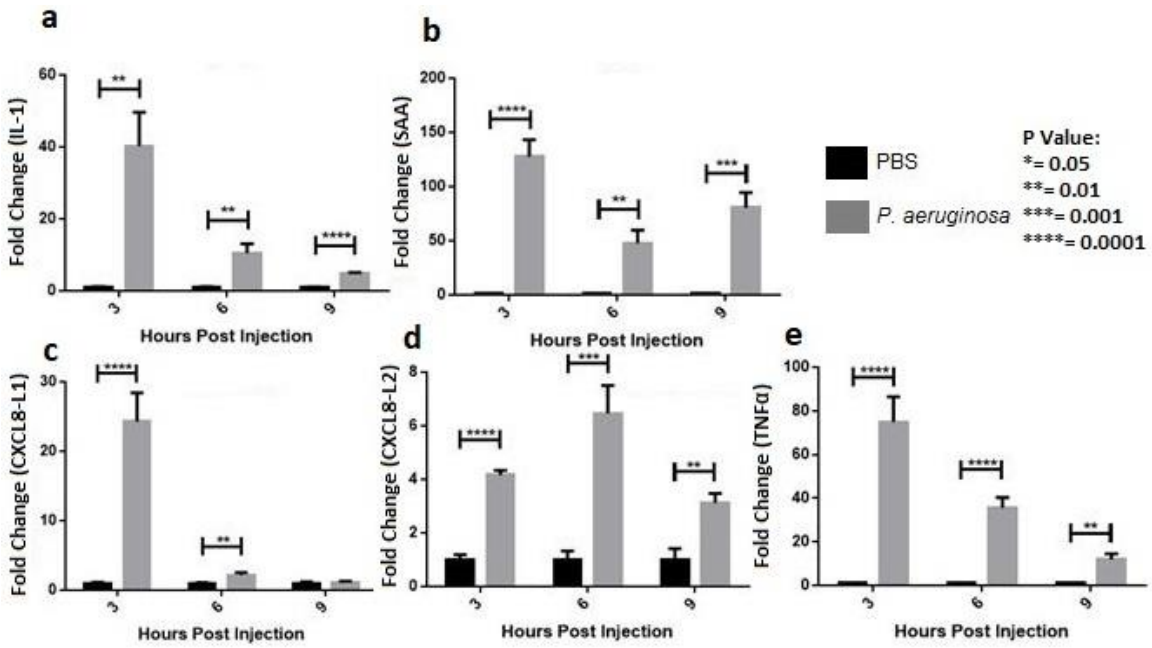
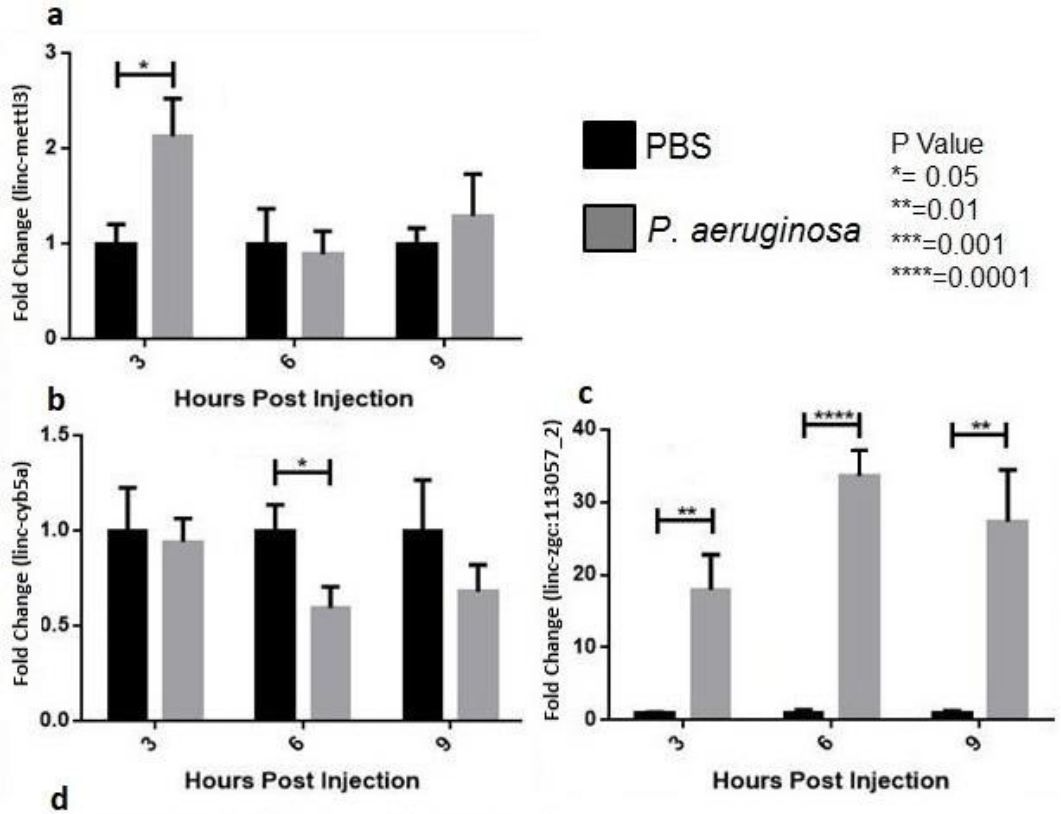


Figure 3.4. A panel of inflammatory cytokines were significantly upregulated in response to *P. aeruginosa* infection. RT-qPCR was used to characterize the innate immune response of zebrafish embryos to *P. aeruginosa* infection at 6 hpi. All assayed cytokines showed statistically significant upregulation at 3 and 6 hpi, characteristic of the innate immune response to bacterial infection. All experiments are representative of 3 biological replicates. Statistical analyses were performed with the unpaired t test. Error bars represent standard error of the mean.

3.3.4. Candidate lincRNAs validate RNA-Seq expression data

After the expression profile of inflammatory cytokines was shown to be characteristic of bacterial infection, the candidate lincRNAs were profiled via RT-qPCR (Figure 3.5.). linc-mettl3 was not significantly differentially expressed by RT-qPCR at 6

hpi, but was found to trend toward downregulation, as was found in the RNA-Seq data. In addition, linc-mettl3 was significantly increased at 3 hpi during *P. aeruginosa* infection, which was contrary to the RNA-Seq data. linc-cyb5a was significantly increased at 6 hpi during infection, with a fold change nearly identical to the RNA-Seq fold change. Lastly, linc-zgc:113057_2 was found to be very strongly upregulated at 3, 6, and 9 hpi, with a greater that 30-fold increase over PBS injection.



d

lincRNA	RNA-Seq Log Fold Change	qPCR Log Fold Change
linc-mettl3	-1.95	-0.89
linc-cyb5a	-0.67	-0.59
linc-zgc:113057_2	2.43	33.63

Figure 3.5. Candidate lincRNAs show disregulation consistent with RNA-Seq. Wild-type zebrafish embryos were infection with 50 CFU/embryo of *P. aeruginosa* at 48 hpi. RNA was isolated at 3, 6. and 9 hpi and then converted to cDNA. RT-qPCR was run to assay the relative expression of the candidate lincRNAs in relation to the RNA-Seq data. (a) linc-mettl3 displays a trend toward downregulation during *P. aeruginosa* infection but this is not significant. In addition, linc-mettl3 is showing the opposite trend

at 3 hpi. (b) linc-cyb5a shows a significant decrease in expression during infection at 6 hpi with a log fold change that is similar to the RNA-Seq log fold change. (c) linc-zgc:113057_2 expression is very strongly upregulated during infection, with a greater than 30-fold induction at 6 hpi. (d) A table showing the log fold change of the RNA-Seq alongside the qPCR log fold change. All experiments are representative of 3 biological replicates. Statistical analyses were performed with the unpaired t test. Error bars represent standard error of the mean.

3.4. Discussion

Expression patterns of lincRNAs previously annotated by Ulitsky et al, Pauli et al, and Kaushik et al. from the RNA-Seq experiment were analyzed to investigate their potential roles in the innate immune response (161, 169, 170). We plotted the expression data from the RNA-Seq experiment using an MA plot and thresholds for P values and FDR, and found five lincRNAs that were differentially expressed during *P. aeruginosa* infection. Three of the candidate lincRNAs were found to be protein-coding genes. One was found to be the protein-coding gene zgc:113057, which interestingly had a neighboring lincRNA called linc-zgc:113057_2. We subsequently added the newly discovered lincRNA to our list of candidate lincRNAs.

The fact that these two genes of similar name are located relatively close to each other on chromosome 7 is interesting and generates new hypotheses for future studies. It would be interesting to determine if linc-zgc:113057_2 is acting as a promoter or co-transcriptional regulator of zgc:113057, which is one of the many potential functions of

lincRNAs (153). It may also prove to be beneficial to find a protein-coding gene with which lincRNAs are associated, since lincRNAs are not well conserved from species to species. This fact makes it difficult to predict that a specific lincRNA in one species will have a similar function or even be present in another species. Although this may be the case for the lincRNAs of interest here, the function of lincRNAs, rather than their sequence or identity, is more likely to be conserved. Therefore, it may be beneficial to show a relationship between a lincRNA and protein-coding gene, because the protein-coding gene is more likely to be conserved (153, 201). In addition, if a relationship between a lincRNA and protein-coding gene can be established it would make it easier to locate a homolog of the lincRNA in another species if the protein-coding gene were to be located first.

To validate the RNA-Seq expression data we aimed to duplicate the *P. aeruginosa* infection portion of the RNA-Seq experiment. To make sure that we produced a proper systemic infection we performed RT-qPCR on a panel of inflammatory cytokines including IL-1, SAA, CXCL8-L1, CXCL8-L2, and TNFα. These cytokines are important in the initial innate immune response to bacterial infection and were all shown to be strongly upregulated in our study at the 6 hpi timepoint, as expected (108, 147, 202, 203). The reduction of CXCL8-L1 expression was also expected as CXCL8-L1 is known to be an early marker of bacterial infection, since it is involved in neutrophil chemotaxis. In CF patients it is known to be consistently upregulated and therefore yields chronic inflammation and neutrophil presence, but in non-CF individuals CXCL8-L1 should eventually decrease after bacterial clearance to maintain proper inflammation levels (6). In addition, CXCL8-L1 may be decreased at 6 and 9 hpi because

its homologue, CXCL8-l2 is more actively upregulated at that time instead of CXCL8-L1. No matter the case, the significant upregulation of the inflammatory cytokines at 6 hpi was measured as expected, confirming the presence of systemic infection in the zebrafish embryos.

Candidate lincRNAs showed significant differential expression at 6 hpi during *P. aeruginosa* infection when compared to PBS injections. linc-mettl3 displayed a trend toward downregulation at 6 hpi, but this failed to be statistically significant. linc-cyb5a was significantly downregulated during *P. aeruginosa* infection and yielded a log fold change that was nearly identical to the RNA-Seq expression data. In addition, linc-zgc:113057_2 was significantly dysregulated at 6 hpi with a greater than 30-fold induction in *P. aeruginosa*-infected zebrafish compared to control zebrafish. These data are exciting because they not only validate the RNA-Seq but they also show a fold-induction greater than expected. This is particularly interesting because linc-RNAs are known for being expressed at very low levels (163, 164). The highest significant expression of lincRNA in the RNA-Seq was linc-zgc:113057_2 with a 2.4 log fold increase over controls. Our finding of a 32-log fold increase of linc-zgc:113057_2 during infection is exciting as it suggests that this is playing a key role in immunity and could potentially lead to improved bacterial therapies.

To further elucidate the function of linc-zgc:113057_2, its expression can be knocked down in the zebrafish prior to challenging the zebrafish with *P. aeruginosa* infection. By utilizing locked nucleic acid (LNA) technology (Exiqon, MD) we can KD linc-zgc:113057_2 and begin to characterize its function. After KD, RNA can be isolated for RT-qPCR analysis and linc-zgc:113057_2 KD can be confirmed. In addition, an

expression profile of cytokines can be analyzed to determine linc-zgc:113057_2 involvement in regulation of cytokine expression. Furthermore, a bacterial burden and respiratory burst assay along with a zebrafish survival experiment could be performed to understand if linc-zgc:113057_2 has a direct effect on bacterial burden, level of ROS, and zebrafish survival. Moreover, linc-zgc:113057_2 can be overexpressed after generation of a clone and the same experiments can be performed. Lastly, an *in situ* hybridization (ISH) can be conducted to determine the spatial expression of linc-zgc:113057_2 in the zebrafish embryo. ISH labels DNA or RNA, allowing visualization of the target through the use of a complementary probe.

In conclusion, five lincRNAs were found to be differentially regulated during *P. aeruginosa* infection in zebrafish embryos in a large-scale RNA-Seq experiment. The RNA-Seq made use of three publications to determine the loci of the lincRNAs (161, 169, 170). Three of the candidate lincRNAs were determined to be protein-coding, but one of them turned out to be zgc:113057, which had linc-zgc:113057_2 as a neighboring gene. This was interesting because lincRNA are known to act as regulators of protein-coding genes and may prove to be useful in locating linc-zgc:113057_2 or a functional orthologue in other species (153, 201). To validate the RNA-Seq, we repeated the infection with *P. aeruginosa* in zebrafish embryos exactly parallel to the method used for the RNA-Seq. We found a characteristic upregulation of inflammatory cytokines in response to bacterial infection. Furthermore, we validated the RNA-Seq expression data with linc-cyb5a and linc-zgc:113057_2 having statistically significant differential expression at 6 hpi. In addition, we found linc-zgc:113057_2 to be more strongly upregulated than previously expected and plan to characterize its function in infection

and immunity. We suggest a series of experiments after KD or overexpression of linc-zgc:113057_2 followed by *P*. aeruginosa infection in zebrafish embryos.

In the early 2000s ncRNA were considered to be junk with no function, but now we are showing here that lincRNAs are being dysregulated during bacterial infection and miRNAs play a role in modulating the respiratory burst response to infection in CFTR morphant zebrafish. It is known that CF is incurable and CF patients have an average life expectancy of only 37 years, which is mostly due to uncontrollable, chronic bacterial infections. Therefore, it is important to continue the discovery and elucidation of ncRNA and their role in immunity to aid in the development of novel therapies for bacterial infection and cystic fibrosis.

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APPENDICES

Appendix A.

Raw bacterial burden data

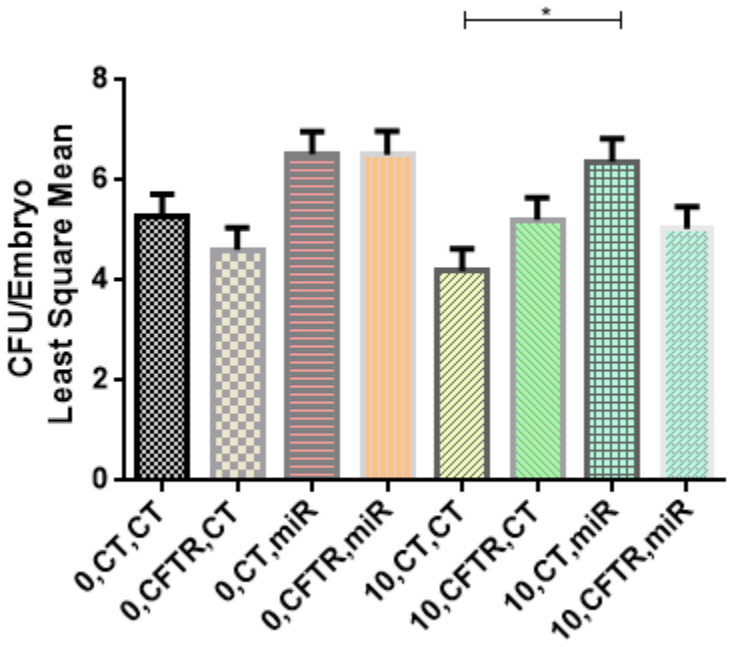

Figure A.1. Bacterial burden biological replicate 1. An increase in bacterial burden is observed in the zebrafish coinjected with control and miRNA-199 MOs compared to control fish. This is the opposite trend that is seen in Figure A.2. between these two experimental groups. These data were analyzed via ANOVA 4-factor analysis using JMP 8.01 (SAS, Cary, NC).

Table A.1. Bacterial burden biological replicate 1 raw data

0,CT,CT	20	120	11	292	132	78	0	50	8	2	25	1	28	408	7	112	168	49	280	352
0,CFTR,CT	44	6	18	37	4	37	58	14	27	13	39	13	27	29	17	5	6	7	508	276
0,CT,miR	20	44	36	15	300	35	5	21	9	9	13	5	3	10	84	9	11	23	25	8
0,CFTR,miR	70	146	16	10	13	308	3	384	12	126	32	148	67	26	68	16	8	40	16	17
10,CT,CT	44	1312	116	232	18	13	824	216	10	308	544	312	54	87	13	33	95	304	6	
10,CFTR,CT	8	172	60	332	31	70	296	14	3	1192	90	139	212	122	436	68	168	78		
10,CT,miR	384	12	192	412	39	119	76	360	61	14	272	16	340	118	248	300	21	14	12	
10,CFTR,miR	97	33	15	11	144	0	34	80	24	200	15	118	11	19	47	110	17	4	96	66

Table A.2. Bacterial burden biological replicate 1 least square mean raw data

Group	Least Sq Mean	Std Error
0,CT,CT	5.278726	0.441538
0,CT,CFTR	4.6140099	0.441538
0,CT,miR	6.5253556	0.453008
0,CFTR,miR	6.5159433	0.465422
10,CT,CT	4.1904388	0.441538
10,CT,CFTR	5.2169868	0.441538
10,CT,miR	6.3775371	0.453008
10,CFTR,miR	5.0285014	0.441538

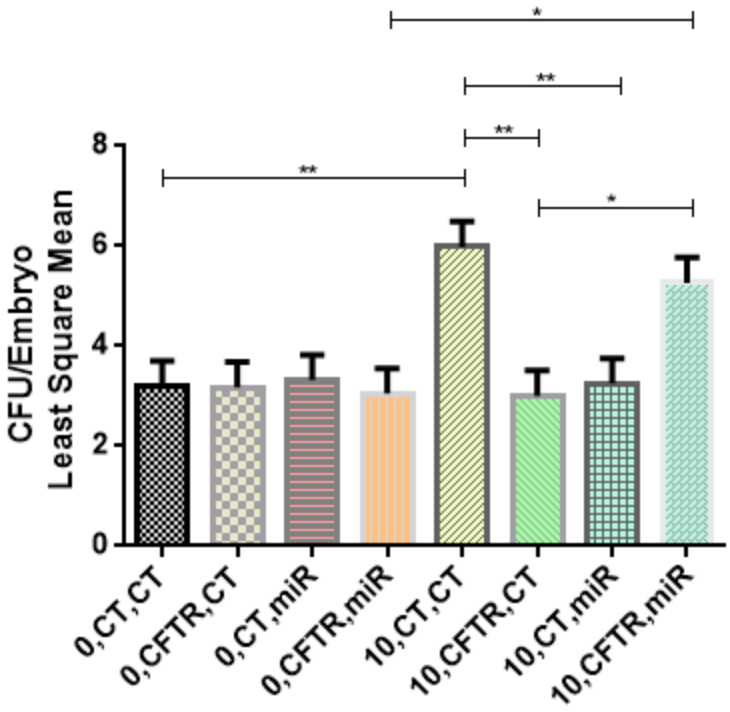

Figure A.2. Bacterial burden biological replicate 2. Arsenic was shown to increase the bacterial burden in zebrafish when compared to zebrafish not exposed to arsenic. In addition, miRNA-199 and control coinjected zebrafish displayed a decreased bacterial burden when compared to control zebrafish. Conversely, zebrafish coinjected with CFTR and miRNA-199 MOs resulted in an increased burden when compared to fish coinjected with CFTR and control MOs. These data were analyzed via ANOVA 4-factor analysis using JMP 8.01 (SAS, Cary, NC).

Table A.3. Bacterial burden biological replicate 2 raw data

0,CT,CT	15	189	2	7	8	7	2	38	4	11	45	7	3	2	5	46	1	3	3	12
0,CFTR,CT	1	107	1	32	4	6	6	2	6	5	1	5	67	12	7	208	1	7	11	8
0,CT,miR	120	10	9	3	322	186	26	174	97	432	480	182	148	15	16	272	248	230	1	14
0,CFTR,miR	240	54	182	107	138	29	4	4	1	1	1	1	2	4	1	1	3	2	1	1
10,CT,CT	6	24	0	13	142	2	3	2	30	2	21	9	5	7	127	82	1	2	5	17
10,CFTR,CT	5	88	24	3	18	1	4	82	1	1	5	3	2	27	14	1	1	5	2	300
10,CT,miR	24	160	1	1	1	10	1	35	1	1	43	1	1	1	1	6	13	169	211	153
10,CFTR,miR	30	7	8	57	210	1	300	400	5	96	62	280	1	56	200	46	1	63	106	50

Table A.4. Bacterial burden biological replicate 2 least square mean raw data

Group	Least Sq Mean	Std Error
0,CT,CT	3.2025257	0.503432
0,CT,CFTR	3.1774156	0.503432
0,CT,miR	3.3204366	0.503432
0,CFTR,miR	3.0468581	0.503432
10,CT,CT	5.9852581	0.503432
10,CT,CFTR	3.0063189	0.503432
10,CT,miR	3.2541185	0.503432
10,CFTR,miR	5.2666642	0.503432

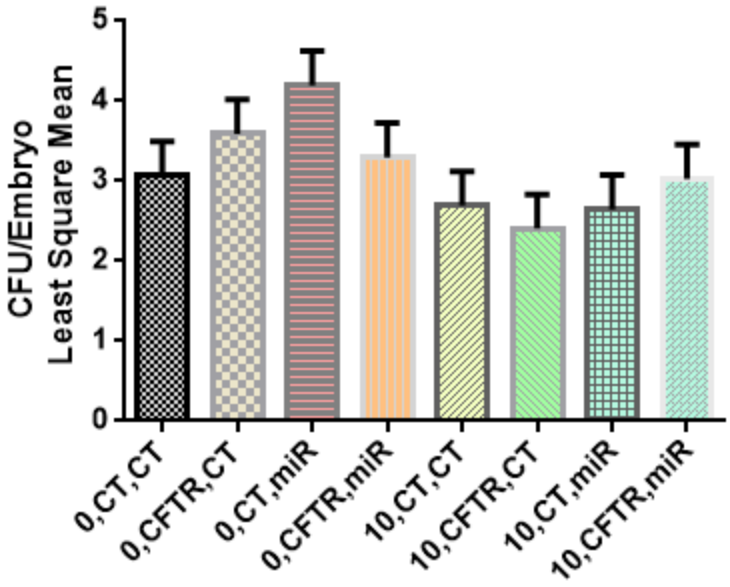

Figure A.3. Bacterial burden biological replicate 3. No significance was observed when analyzing biological replicate 3. These data were analyzed via ANOVA 4-factor analysis using JMP 8.01 (SAS, Cary, NC).

Table A.5. Bacterial burden biological replicate 3 raw data

0,CT,CT	2	57	12	1	2	6	3	15	87	2	1	3	5	8	2	15	38	2	30	24
0,CFTR,CT	3	76	110	3	4	17	128	2	3	2	56	4	1	2	15	6	76	94	16	2
0,CT,miR	2	3	14	1	2	26	23	8	35	11	6	3	10	3	5	2	1	6	1	9
0,CFTR,miR	1	2	2	3	1	2	1	2	2	67	216	8	1	2	6	1	20	27	2	1
10,CT,CT	104	2	1	47	1	2	64	9	2	57	118	18	83	99	94	60	7	4	2	68
10,CFTR,CT	2	1	186	2	2	3	3	10	9	3	4	9	5	6	2	2	66	288	36	73
10,CT,miR	5	2	4	1	2	5	2	6	2	34	3	4	9	3	3	52	27	2	41	1
10,CFTR,miR	7	2	4	2	1	2	3	2	41	101	17	129	30	290	2	2	2	2	2	2

Table A.6. Bacterial burden biological replicate 3 least square mean raw data

Group	Least Sq Mean	Std Error
0,CT,CT	3.0694181	0.429451
0,CT,CFTR	3.5895205	0.429451
0,CT,miR	4.197565	0.429451
0,CFTR,miR	3.2974581	0.429451
10,CT,CT	2.6953736	0.429451
10,CT,CFTR	2.4060352	0.429451
10,CT,miR	2.6462376	0.429451
10,CFTR,miR	3.028383	0.429451

Appendix B.

Raw respiratory burst data

Table B.1. Respiratory burst assay biological replicate 1 raw data.

0,CT,CT	0,CFTR,CT	0,CT,miR	0,CFTR,miR	10,CT,CT	10,CFTR,CT	10,CT,miR	10,CFTR,miR
134721	84508	138261	143439	131124	112946	95291	136231
123465	82189	129472	135356	135125	133804	129927	117730
116588	99336	147343	121231	129091	130975	121075	117377
134192	96576	142755	146189	135014	128192	135623	121766
106956	81338	143733	124540	136830	130847	127883	136974
114055	93264	123389	1613	133927	136855	73484	130160
135102	97979	143793	126219	131473	139109	131148	130555
126116	80315	136104	109631	143511	138583	124739	135754
137023	88626	125708	143123	141987	128371	85426	108415
131532	93985	136948	128091	137406	127645	135901	127725
128319	84471	137844	149378	129603	128891	127296	112935
140731	58900	158429	109285	143778	116239	126882	102640
127476	90692						
135134	11898						
108856	87830						
120626	88010						

Table B.2. Respiratory burst assay biological replicate 1 least square mean raw data.

Group	Least Sq Mean	Std Error
0,CT,CT	126305.75	4693.446
0,CT,CFTR	82494.81	4693.446
0,CT,miR	138648.25	5419.524
0,CFTR,miR	119841.25	5419.524
10,CT,CT	135739.08	5419.524
10,CT,CFTR	129371.42	5419.524
10,CT,miR	117889.58	5419.524
10,CFTR,miR	123188.5	5419.524

Table B.3. Respiratory burst assay biological replicate 2 raw data.

0,CT,CT	0,CFTR,CT	0,CT,miR	0,CFTR,miR	10,CT,CT	10,CFTR,CT	10,CT,miR	10,CFTR,miR
71235	7189	60837	63205	17839	852	73744	63964
64499	56109	71949	65950	56500	69401	64921	58955
62532	55431	54116	66778	58571	76043	32767	63156
68561	64050	50882	62370	68245	69389	69239	78818
56671	65181	66801	64536	56918	48063	70991	49031
62053	60991	57949	37954	62005	64592	80208	68162
60345	65716	115710	80979	51211	21084	73106	71056
60508	69649	33726	6013	51996	12537	76862	73345
59806	58005	106515	9191	61156	64909	72212	59835
43865	62237	95554	26809	64733	84089	65737	66240
56875	51861	26105	27570	61649	4027	59681	70548
63469	59221	21104	12488	66411	5524	70110	68060

Table B.4. Respiratory burst assay biological replicate 2 least square mean raw data.

Group	Least Sq Mean	Std Error
0,CT,CT	60868.25	5895.511
0,CT,CFTR	56303.333	5895.511
0,CT,miR	63437.333	5895.511
0,CFTR,miR	43653.583	5895.511
10,CT,CT	56436.167	5895.511
10,CT,CFTR	43375.833	5895.511
10,CT,miR	67464.833	5895.511
10,CFTR,miR	65930.833	5895.511

Table B.5. Respiratory burst assay biological replicate 3 raw data.

0,CT,CT	0,CFTR,CT	0,CT,miR	0,CFTR,miR	10,CT,CT	10,CFTR,CT	10,CT,miR	10,CFTR,miR
180803	21327	138197	63256	133040	129374	24269	180509
134591	127123	154383	145717	96481	76754	22668	146907
126025	127807	159530	144293	120288	23351	46633	10690
156758	135317	140634	144390	144969	16230	13110	147678
148337	44839	138043	174428	149802	22334	11290	155253
148327	55349	179605	142388	115499	69404	28943	180123
116703	85724	55481	49273	101361	12411	18283	115823
133000	152302	68361	33275	110242	83681	41890	148906
157502		20997	78982	147260	56286		136164
155110			56580	138723			88983
155869				171110			
150821				109048			

Table B.6. Respiratory burst assay biological replicate 3 least square mean raw data.

Group	Least Sq Mean	Std Error
0,CT,CT	146987.17	11433.66
0,CT,CFTR	93723.5	14003.31
0,CT,miR	117247.89	13202.45
0,CFTR,miR	103258.2	12524.94
10,CT,CT	128151.92	11433.66
10,CT,CFTR	54425	13202.45
10,CT,miR	25885.75	14003.31
10,CFTR,miR	131103.6	12524.94

Appendix C.

Raw RT-qPCR Cq data

Table C.1. 18s RNA raw Cq values

Time (hpi)	P.a. or PBS injected	Replicate #1 Cq	Replicate #2 Cq	Replicate #3 Cq
3	PBS	22.57	23.01	23.88
3	PBS	22.22	22.68	21.28
3	PBS	21.91	22.36	23.03
3	PBS	21.74	23.08	21.74
3	PBS	21.86	22.75	21.21
6	PBS	22.37	22.23	22.20
6	PBS	22.76	22.60	21.95
6	PBS	24.20	22.35	20.92
6	PBS	23.84	22.17	21.19
6	PBS	22.28	21.94	21.64
9	PBS	22.54	23.10	21.92
9	PBS	23.03	24.04	21.39
9	PBS	21.89	22.35	21.99
9	PBS	21.93	22.35	20.82
9	PBS	26.16	23.78	22.55
3	P.a.	23.15	22.98	22.26
3	P.a.	23.02	22.33	21.20
3	P.a.	23.85	21.87	20.86
3	P.a.	23.19	21.60	20.98
3	P.a.	23.59	21.23	20.80
6	P.a.	22.88	24.53	21.44
6	P.a.	22.36	22.20	21.29
6	P.a.	22.42	22.29	22.15
6	P.a.	22.14	21.70	20.78
6	P.a.	22.39	25.79	22.08
9	P.a.	23.21	22.24	21.94
9	P.a.	22.99	22.76	21.02
9	P.a.	21.68	24.54	20.80
9	P.a.	21.82	22.18	21.38
9	P.a.	24.92	22.36	23.53

Table C.2. IL-1 RNA raw Cq values

Time (hpi)	P.a. or PBS injected	Replicate #1 Cq	Replicate #2 Cq	Replicate #3 Cq
3	PBS	32.32	31.20	30.28
3	PBS	32.29	30.60	30.32
3	PBS	31.09	30.56	31.32
3	PBS	32.21	30.18	31.70
3	PBS	31.45	31.17	31.19
6	PBS	32.61	34.04	31.34
6	PBS	33.24	31.77	32.03
6	PBS	34.15	32.16	31.11
6	PBS	33.02	30.56	31.50
6	PBS	32.59	32.61	35.09
9	PBS	32.20	32.57	30.81
9	PBS	35.00	32.59	30.09
9	PBS	33.08	32.69	30.09
9	PBS	33.63	33.00	31.53
9	PBS	35.71	34.73	33.27
3	P.a.	27.98	26.59	26.25
3	P.a.	28.27	26.89	25.93
3	P.a.	27.55	27.58	26.03
3	P.a.	28.64	26.64	27.34
3	P.a.	28.97	26.22	26.48
6	P.a.	30.04	31.07	28.45
6	P.a.	29.91	28.54	29.05
6	P.a.	29.13	27.30	27.72
6	P.a.	31.09	27.79	27.13
6	P.a.	29.65	37.12	30.01
9	P.a.	32.22	28.83	28.93
9	P.a.	28.47	27.37	28.76
9	P.a.	28.15	27.24	28.52
9	P.a.	28.53	28.55	28.56
9	P.a.	34.21	25.33	29.27

Table C.3. CXCL8-L1 RNA raw Cq values

Time (hpi)	P.a. or PBS injected	Replicate #1 Cq	Replicate #2 Cq	Replicate #3 Cq
3	PBS	25.86	28.63	29.22
3	PBS	25.98	28.89	28.24
3	PBS	26.02	28.49	28.42
3	PBS	26.11	27.32	28.68
3	PBS	26.64	28.25	28.03
6	PBS	26.40	28.71	29.22
6	PBS	26.98	28.14	28.24
6	PBS	27.95	28.37	28.42
6	PBS	27.80	28.15	28.68
6	PBS	27.56	27.95	28.03
9	PBS	25.36	28.31	28.46
9	PBS	26.94	28.91	28.27
9	PBS	25.68	29.05	28.37
9	PBS	25.87	27.93	28.18
9	PBS	27.14	28.67	30.68
3	P.a.	23.69	25.10	24.87
3	P.a.	30.04	24.64	24.98
3	P.a.	23.48	25.24	25.41
3	P.a.	24.66	24.36	24.98
3	P.a.	24.53	24.63	24.54
6	P.a.	24.74	26.16	26.61
6	P.a.	24.42	26.11	25.46
6	P.a.	25.60	25.70	26.30
6	P.a.	26.49	25.56	24.34
6	P.a.	25.39	26.71	27.52
9	P.a.	26.28	26.76	26.63
9	P.a.	22.82	25.38	26.57
9	P.a.	22.71	24.53	26.43
9	P.a.	23.14	25.60	26.84
9	P.a.	24.16	24.48	26.62

Table C.4. CXCL8-L2 RNA raw Cq values

Time (hpi)	P.a. or PBS injected	Replicate #1 Cq	Replicate #2 Cq	Replicate #3 Cq
3	PBS	31.52	32.38	31.82
3	PBS	30.74	31.02	31.56
3	PBS	28.85	32.03	31.69
3	PBS	30.88	31.61	31.98
3	PBS	30.02	32.37	31.81
6	PBS	30.31	32.02	31.60
6	PBS	31.29	31.36	34.32
6	PBS	32.33	32.68	32.10
6	PBS	31.15	32.50	32.67
6	PBS	31.35	32.69	33.22
9	PBS	31.99	32.23	31.65
9	PBS	32.84	33.70	32.01
9	PBS	31.66	32.30	32.36
9	PBS	30.61	32.20	31.03
9	PBS	35.19	32.18	35.77
3	P.a.	28.53	30.17	31.30
3	P.a.	28.29	30.02	31.09
3	P.a.	28.77	30.95	31.45
3	P.a.	28.52	30.77	31.49
3	P.a.	30.23	29.72	30.06
6	P.a.	25.35	28.71	29.69
6	P.a.	26.47	29.13	28.27
6	P.a.	26.21	28.28	28.43
6	P.a.	28.07	27.76	29.36
6	P.a.	26.43	30.33	31.34
9	P.a.	31.04	29.01	30.54
9	P.a.	27.06	30.41	29.40
9	P.a.	28.27	28.20	29.46
9	P.a.	28.04	27.47	30.00
9	P.a.	28.32	27.27	30.05

Table C.5. SAA RNA raw Cq values

Time (hpi)	P.a. or PBS injected	Replicate #1 Cq	Replicate #2 Cq	Replicate #3 Cq
3	PBS	34.33	31.49	33.91
3	PBS	33.92	32.61	30.02
3	PBS	31.90	34.08	31.47
3	PBS	31.95	29.62	29.60
3	PBS	32.87	30.87	30.35
6	PBS	34.48	34.08	30.17
6	PBS	34.78	33.10	31.25
6	PBS	35.64	30.76	29.64
6	PBS	35.27	32.63	31.21
6	PBS	36.06	35.38	29.32
9	PBS	36.87	31.58	31.37
9	PBS	36.26	35.33	30.85
9	PBS	34.28	30.74	31.21
9	PBS	34.57	31.46	29.12
9	PBS	35.49	35.36	34.37
3	P.a.	27.16	27.49	26.63
3	P.a.	27.09	26.20	27.12
3	P.a.	27.22	27.14	26.85
3	P.a.	28.94	26.78	26.63
3	P.a.	27.91	26.82	26.90
6	P.a.	26.48	26.13	26.61
6	P.a.	26.75	27.66	26.52
6	P.a.	26.47	26.40	26.76
6	P.a.	27.07	26.85	26.63
6	P.a.	26.37	33.90	26.55
9	P.a.	27.60	26.30	25.19
9	P.a.	26.37	25.81	26.55
9	P.a.	27.05	25.79	26.62
9	P.a.	26.54	25.38	27.65
9	P.a.	28.86	25.28	27.41

Table C.6. TNFα RNA raw Cq values

Time (hpi)	P.a. or PBS injected	Replicate #1 Cq	Replicate #2 Cq	Replicate #3 Cq
3	PBS	33.26	36.14	34.49
3	PBS	33.41	35.35	33.24
3	PBS	31.85	34.26	35.37
3	PBS	39.96	33.64	33.17
3	PBS	33.69	36.16	34.14
6	PBS	34.71	36.22	36.14
6	PBS	34.39	35.87	34.13
6	PBS	33.89	35.06	35.08
6	PBS	34.28	35.12	35.49
6	PBS	35.20	35.54	35.49
9	PBS	35.60	35.24	36.37
9	PBS	34.95	39.34	37.71
9	PBS	34.17	35.37	37.71
9	PBS	34.55	35.30	37.71
9	PBS	33.33	38.40	37.71
3	P.a.	29.44	30.51	30.51
3	P.a.	28.98	30.07	30.54
3	P.a.	28.30	30.23	30.69
3	P.a.	29.85	30.32	30.10
3	P.a.	27.43	29.47	30.09
6	P.a.	28.83	31.21	32.55
6	P.a.	29.69	31.75	29.75
6	P.a.	29.91	31.23	31.68
6	P.a.	30.48	31.47	29.81
6	P.a.	28.28	31.55	31.60
9	P.a.	33.43	29.58	31.12
9	P.a.	35.82	29.63	31.08
9	P.a.	30.30	29.04	30.08
9	P.a.	29.70	30.26	30.42
9	P.a.	31.71	30.02	32.27

Table C.7. linc-mettl3 RNA raw Cq values

Time (hpi)	P.a. or PBS injected	Replicate #1 Cq	Replicate #2 Cq	Replicate #3 Cq
3	PBS	28.99	28.89	27.16
3	PBS	28.80	28.15	26.61
3	PBS	28.23	28.06	27.34
3	PBS	29.01	28.43	28.33
3	PBS	28.48	28.64	27.43
6	PBS	28.88	28.19	26.60
6	PBS	29.34	28.37	27.06
6	PBS	29.40	28.31	26.69
6	PBS	29.06	28.04	25.14
6	PBS	28.18	27.70	32.34
9	PBS	28.32	27.94	27.03
9	PBS	29.94	28.94	26.07
9	PBS	28.18	27.93	26.07
9	PBS	27.40	27.59	26.30
9	PBS	29.17	28.53	28.15
3	P.a.	28.03	27.17	25.59
3	P.a.	29.06	27.28	24.87
3	P.a.	28.99	28.34	25.85
3	P.a.	29.04	27.36	27.23
3	P.a.	29.47	25.62	27.06
6	P.a.	27.04	28.82	26.37
6	P.a.	28.02	27.44	27.45
6	P.a.	27.71	26.71	25.50
6	P.a.	28.49	27.31	24.61
6	P.a.	27.55	35.69	27.47
9	P.a.	30.22	27.63	27.23
9	P.a.	26.29	25.62	25.73
9	P.a.	26.91	26.98	25.22
9	P.a.	26.29	26.52	26.66
9	P.a.	32.03	25.07	28.93

Table C.8. linc-cyb5a RNA raw Cq values

Time (hpi)	P.a. or PBS injected	Replicate #1 Cq	Replicate #2 Cq	Replicate #3 Cq
3	PBS	29.15	28.84	31.57
3	PBS	30.24	28.28	31.64
3	PBS	29.49	27.73	32.20
3	PBS	30.00	28.10	34.04
3	PBS	29.29	28.12	31.52
6	PBS	29.09	28.60	32.11
6	PBS	29.54	30.31	32.67
6	PBS	30.36	28.39	31.42
6	PBS	30.49	29.19	33.13
6	PBS	30.16	29.38	32.26
9	PBS	30.03	28.28	31.89
9	PBS	31.26	28.23	31.64
9	PBS	30.18	28.31	33.25
9	PBS	29.29	28.31	33.21
9	PBS	31.46	31.07	34.32
3	P.a.	29.45	27.96	35.03
3	P.a.	29.52	27.63	31.81
3	P.a.	30.33	27.61	31.86
3	P.a.	30.99	28.03	32.84
3	P.a.	30.70	27.47	32.93
6	P.a.	29.73	28.16	33.47
6	P.a.	29.75	28.78	34.01
6	P.a.	29.51	28.43	32.22
6	P.a.	30.06	29.06	32.49
6	P.a.	29.58	29.10	33.87
9	P.a.	31.71	30.38	32.62
9	P.a.	29.93	29.17	32.98
9	P.a.	30.38	28.43	33.16
9	P.a.	30.10	30.33	33.04
9	P.a.	29.89	30.64	35.47

Table C.9. linc-zgc:113057_2 RNA raw Cq values

Time (hpi)	P.a. or PBS injected	Replicate #1 Cq	Replicate #2 Cq	Replicate #3 Cq
3	PBS	30.57	30.27	30.10
3	PBS	30.36	30.39	29.99
3	PBS	30.31	30.20	29.34
3	PBS	30.62	30.07	29.57
3	PBS	31.26	29.89	29.42
6	PBS	30.46	30.05	29.84
6	PBS	30.26	29.68	30.99
6	PBS	29.32	29.51	28.70
6	PBS	28.95	28.51	29.17
6	PBS	30.36	31.01	31.59
9	PBS	30.78	31.05	30.95
9	PBS	30.83	29.56	30.06
9	PBS	30.83	34.38	29.47
9	PBS	30.18	29.33	29.74
9	PBS	29.98	29.64	31.26
3	P.a.	27.27	27.05	26.96
3	P.a.	26.56	26.02	27.46
3	P.a.	26.97	26.27	27.16
3	P.a.	28.31	28.09	27.76
3	P.a.	28.04	27.70	26.13
6	P.a.	25.03	24.66	27.38
6	P.a.	25.15	24.99	28.86
6	P.a.	24.10	23.75	26.50
6	P.a.	24.73	24.22	28.21
6	P.a.	24.45	24.25	28.10
9	P.a.	24.89	25.64	26.96
9	P.a.	27.18	24.52	27.81
9	P.a.	24.76	26.85	28.56
9	P.a.	24.67	24.41	27.52
9	P.a.	24.67	24.39	28.67

BIOGRAPHY OF THE AUTHOR

Jacob R. Longfellow was born in Lewiston, Maine on March 31st, 1992. He and his extended family live in Farmingdale, Maine, where he grew up surrounded by family. He graduated from Hall-Dale High School in 2010 in the top ten of his class. In the fall of 2010 Jacob began his higher education at the University of Maine where he would join Beta Theta Pi fraternity and quickly rise to president. He graduated with Honors on May 10th, 2014 with a Bachelor of Science in Microbiology. He was accepted into the Master of Science program in Microbiology at the University of Maine in the fall of 2014 and subsequently graduated in the summer of 2017. On May 17, 2017 Jacob was accepted into Medical school at The University of New England School of Osteopathic Medicine. Jacob is a candidate for the Master of Science degree in Microbiology from the University of Maine in August 2017.