Regulation of microRNA-199 Upon Pseudomonas Aeruginosa Infection in Zebrafish

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REGULATION OF MICRORNA-199 UPON *PSEUDOMONAS AERUGINOSA* INFECTION

IN ZEBRAFISH

by

Eliot Rivers Gagné

A Thesis Submitted in Partial Fulfillment of the Requirements for a Degree with Honors (Biochemistry)

The Honors College

The University of Maine

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Abstract:

The innate immune response is vital in an organism’s ability to fight infection. Aspects of the innate immune response including inflammation, phagocyte populations, and the production of reactive oxygen species (ROS) must be closely regulated in order to effectively eliminate pathogenic entities. Dysregulation of the innate immune response can cause various pathologies, including chronic infection, autoimmunity, and cancer. Recent studies have shown that microRNAs (miRs) play a regulatory and “fine-tuning” role in the innate immune system of the Zebrafish. We have found that the expression of miR-199 is significantly upregulated upon stimulation of the Zebrafish innate immune system with *Pseudomonas aeruginosa* infection. Knocking-down the expression of miR-199 using oligonucleotide morpholino injection led to a significant decrease in bacterial burden of fish infected with *P. aeruginosa* as well as a significant increase in overall survival. Overexpression of miR-199 using a mature miR-199 duplex led to a decrease in overall reactive oxygen species (ROS) within an infected organism. Recent data also suggest that direct targeting of Src family kinase (SFK) *Lyn* by miR-199 may be responsible for the immunosuppressant characteristics of this specific microRNA.
Acknowledgements:

First of all, I would like to especially thank my thesis advisor Dr. Carol Kim. I would also like to thank all of the members of my thesis committee which includes Dr. Sally Molloy, Dr. Melissa Ladenheim, Dr. Robert Gundersen, and Dr. Paul Millard. A special thanks is also owed to all of my graduate mentors which include Dawn Sullivan, Richard Luc and Campbell Miller. Furthermore, I am thankful for all of the members of the Kim lab for providing a comfortable and thought provoking environment. Finally, I want to thank my family and friends for being supportive of my dreams and standing with me throughout my undergraduate career.
Table of Contents:

Introduction.............................................................................................................1

Literature Review....................................................................................................2

Overview of Immunity.............................................................................................2

Innate Immunity....................................................................................................4

_Pseudomonas aeruginosa_.....................................................................................8

Zebrafish as a Model Organism............................................................................10

MicroRNAs..........................................................................................................13

MiR Biogenesis....................................................................................................13

Targeting of miRs.................................................................................................16

MiRs in Immunity.................................................................................................17

MiR-199 family....................................................................................................20

Functional Roles for miR-199 in Human Health................................................21

Materials & Methods...........................................................................................23

Results..................................................................................................................28

Discussion.............................................................................................................33

Works Cited..........................................................................................................36

Authors Biography...............................................................................................44
**Introduction:**

As human beings, we are exposed to a wide variety of pathogenic entities on a regular basis. Whether those pathogens are of viral or bacterial origin, we rely on our immune system to clear the infection. The human immune system is composed of two branches, the innate immune response and the adaptive immune response. The innate immune response is a rapid and non-specific response to a wide variety of invading pathogens and is active in humans within the first 12 hours of infection. The adaptive immune response is a more specific response and is associated with antibody production, memory T cells and memory B cells and is active 1-5 days post infection in humans. While both branches of immunity are worthy of investigation, our efforts are focused on the innate branch of the immune system due to the fact that it is an organism’s first line of defense against an invading pathogen and is integral in the establishment of a robust and efficient overall immune response.

The zebrafish (*Danio rerio*) has become a valuable model organism for studying the innate immune system due to the fact that within the first 4-6 weeks of development the fish solely possesses an innate immune response. The zebrafish is also a valuable model in which to study chronic diseases that dysregulate the immune system such as Cystic Fibrosis. Cystic Fibrosis or CF is a multi-organ disease that is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The CFTR gene is conserved within the zebrafish genome and makes this organism a valid model to study CF. CF is known to be immunocompromising and is complicated by bacterial infections. These bacterial infections are commonly caused by *P. aeruginosa* and can become chronic and persistent, leading to increased morbidity and mortality. Our research is directed towards the gene-environment interactions that are associated with CF disease and its progression.
To investigate this complex gene-environment interaction in the context of innate immune system a large RNAseq/small RNAseq analysis was completed. The RNAseq analysis cross-examined genomes from zebrafish depleted of CFTR via oligonulceotide morpholino, exposure to environmentally relevant As, and infected by *P. aeruginosa*. The data retrieved from this large data set identified the dysregulation of miR-199 in As exposed and CFTR depleted samples, specifically during infection or stimulation of the innate immune response. miRs are known to play important roles in many human pathologies and have recently been implicated in the immune system. Therefore, the focus of this paper is to identify the role of miR-199 in the context of innate immunity.

**Literature Review:**

**Overview of Immunity**

Microorganisms are present throughout the environment and can exist in soil, water, on abiotic surfaces, and as commensal species within the normal human flora. Commensal bacteria are critical in the physical barriers of the host as they function to protect the host from external pathogens. These bacteria and other environmental microbes which are typically non-infectious, may become pathogenic following perturbation to the host\(^1\). Wounds, disease, and previous infections are potential mechanisms by which an opportunistic pathogen might cause disease. Opportunistic pathogens and other foreign invaders are constantly challenging host survival. Humans and upper level vertebrates possess an immune system composed of two main branches, the innate immune system and the adaptive immune system. All multicellular organisms possess an innate immune system. This rapid and immediate response is an organism’s first line of defense and is established immediately upon exposure to a foreign pathogenic entity. The innate
immune response is also characterized as a non-specific response and is therefore capable of recognizing and eliminating a broad range of invaders.

Nearly 500 million years ago vertebrates, jawed fish and jawless fish, such as lampreys and hagfish, evolved a secondary branch of immunity, known as the adaptive immune system. The adaptive immune system, distinguishes itself from innate immunity in its ability to recognize specific antigens and its capacity to form immunologic memory. Memory recalls specific pathogens upon subsequent exposure and enables a quicker, more specific response as compared to the response to the primary infection. In contrast to the immediate nature of the innate immune response, mounting an adaptive immune response takes significantly more time. In humans, primary infections require 7-14 days to elicit an effective adaptive immune response. Cellular components of the adaptive immune system include T-cells and B-cells. T-cells mature in the thymus and act via cell-mediated immunity, sensing “self” from “non-self” on the surface of antigen presenting cells (APCs). B-cells mature in the bone marrow and participate in humoral immunity by recognizing foreign antigens and undergoing clonal selection, expansion, and differentiation into memory cells or plasma cells. The innate and adaptive immune systems must work synergistically to defend the host; therefore, an effective adaptive immune system requires a strong innate immune response.
Figure 1: Basic overview of both branches of the immune system and their associated cell types (Fisher et. al 2011).

**Innate Immunity**

The innate immune system is evolutionarily intrinsic to eukaryotes and acts as the first line of defense against invading pathogenic entities. Innate immunity utilizes temperature, pH, chemical inhibitors, as well as physical, inflammatory, and phagocytic barriers to protect the host. The primary functions of the innate immune system are to recruit immune cells to the site of infection, establish an inflammatory response, and activate the adaptive immune system. If any of these functions are dampened or inhibited, the infected organism is at greater risk for complication or death associated with infection.

When a pathogen successfully bypasses the physical barriers of a host, such as the skin and mucosal layers, pathogen-associated molecular patterns (PAMPs) expressed on its surface
are recognized by host pattern recognition receptors (PRRs) found on various host cell types. Some common PAMPs include lipopolysaccharide (LPS), lipoproteins, bacterial double stranded DNA (dsDNA) (i.e. CpG motifs), viral single stranded RNA (ssRNA), and viral dsRNA. These PAMPs enable the innate immune system to distinguish self from non-self and mount an appropriate response. PRRs recognize foreign molecules through two classes of receptors, membrane bound and cytosolic. Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) represent the membrane bound receptors, whereas Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) are cytosolic receptors. Each of these receptors functions through recognition of different types of PAMPs and activate downstream signaling components of the innate immune system.

The TLR family is among the most widely studied of the PRRs. This transmembrane protein family consists of numerous homologues and binds particular PAMPs from bacteria, viruses, and fungi. Receptor-ligand binding leads to complex downstream signaling and the production of proinflammatory cytokines as well as co-stimulatory molecules essential for the activation of the adaptive immune system. TLR signaling upregulates the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which regulates the immune response to infection by inducing expression of cytokines such as tumor necrosis factor α (TNF-α), IL1, and IL6. Cytokines play an essential role in the inflammatory response as their signals lead to recruitment of immune cells, vasodilation, temperature increase, and further propitiate the inflammatory response. Inflammation is one characteristic of the innate immune system that limits the spread of pathogens via the recruitment of immune cells to the site of infection.

Activation and migration of immune cells such as neutrophils, macrophages, eosinophils,
mast cell, and dendritic cells to the site of infection is integral to pathogen elimination.

Eosinophils and mast cells contain cytoplasmic granules with inflammatory and antimicrobial components. Stimulation of these cells by foreign antigen induces the release of granule contents into extracellular space to combat infection. Dendritic cells play an integral role in immunity in that they phagocytose foreign pathogens and signal the adaptive immune response via antigen presentation. These antigen presenting dendritic cells serve as a link between the two branches of immunity. Macrophages and neutrophils are professional phagocytes that internalize foreign bodies and apoptotic cell corpses, ultimately leading to their destruction. Phagocytosis is a receptor-mediated process that employs PAMP-PRR interactions to internalize pathogens. PAMP recognition by phagocytic cells involves receptors such as, TLRs, mannose, CD14, and scavenger receptor A (CD204). Once foreign bodies or debris are internalized, a vacuole, termed phagosome, undergoes a series of fusion and fission events with components of the endocytic pathway. The maturation of the phagosome involves late endosome and lysosome fusion, leading to mature phagosomes and phagolysosomes, respectively. As phagolysosomes mature, the vacuole becomes acidic and contains anti-microbial peptides necessary for the destruction of engulfed pathogens. The mature phagolysosome contains highly oxidizing components associated with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The release of these oxidizing components within the immune cells contributes to an immune phenomenon known as a respiratory burst. The ability of a host to produce a respiratory burst suggests the capability of mounting a robust innate immune response.

Reactive oxygen species (ROS) and nitrogen species (RNS) are involved in the highly oxidizing environment of the phagolysosome and are essential for the clearance of bacterial and fungal infections via respiratory burst. ROS includes superoxide radicals, hydrogen peroxide,
and hydrochlorous acid, all of which are formed by pre-existing components of NADPH oxidase\textsuperscript{8}. Although both ROS and RNS destroy pathogens through cell membrane destruction, DNA damage, and protein disturbance, the formation of nitric oxide must be catalyzed, as it is not a pre-existing component of NADPH oxidase. This catalytic activity is controlled by inducible nitric oxide synthase 2 (iNOS). iNOS gene expression is activated by proinflammatory cytokine stimulation in immune cells. iNOS activity yields NO\textsuperscript{−}, a toxic radical, that can readily react with ROS to produce various toxic reactive nitrogen intermediates (RNI), leading to pathogen elimination. Additionally, a recent study by Yoo et al suggests that the Src family kinase (SFK) Lyn is involved in the recruitment and activation of neutrophils in response to stimulation of the innate immune system and the production of ROS. (Yoo et al)

The innate immune system, with its anatomic, physiologic, inflammatory, and phagocytic barriers, provides essential protection of organisms. The activation and suppression of the innate immune response requires tight regulation of immune-related genes and cytokines. Abnormal gene expression in response to foreign pathogens affects different facets of the innate immune response. In some cases, gene dysregulation can enhance disease progression and lead to chronic infections. Gene regulation is under the control of a wide variety of signaling cascades, enzymes, transcription factors and microRNAs (miR). MiRs have recently come to light as key players in the control of biological and physiological processes. These biological regulators influence gene expression post-transcriptionally through transcript degradation, translational repression, and in rare cases, translational activation\textsuperscript{10}. Gene regulation by miRs has been shown to serve a functional role in human diseases, such as immune related diseases, cardiovascular disease, Alzheimer’s disease, and many cancers\textsuperscript{11–15}. The ability to fight infection relies on strict gene regulation; this relatively new class of regulators may help elucidate the
mechanism by which genes are being regulated during immunity, possibly contributing to new gene therapies.

*Pseudomonas aeruginosa*

*P. aeruginosa*, an opportunistic human pathogen, is one of the leading causes of hospital infections and is responsible for increased morbidity and mortality in Cystic Fibrosis (CF) patients. *P. aeruginosa* causes both acute and chronic infections in a wide diversity of animals and plants. This Gram-negative bacterium can be found naturally in water, soil, and as commensal skin flora. *P. aeruginosa* typically infects immunocompromised hosts; however, clinically significant infections, e.g. keratitis, skin, respiratory tract, and bloodstream infections, have been documented in otherwise healthy individuals. The ability for *P. aeruginosa* to evade host immunity, as well as its intrinsic resistance to antibiotics, makes this bacterium difficult to treat and eradicate as a human pathogen.

*P. aeruginosa* contains several surface structures including a lipopolysaccharide (LPS) outer membrane, single polar flagellum, Type IV pili, and chaperone pili, all of which are recognized by the immune system. LPS, predominantly the lipid A component and a structural variation of the O-antigen, is critical in *P. aeruginosa* infections as it impacts both virulence and host immunity. LPS is recognized by TLR4 in mammals to stimulate an innate immune response. TLR4-LPS interaction commences a downstream signaling cascade that acts notably through NF-kB to produce pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α. The NF-kB pathway can also be activated by TLR5 and TLR2 signaling in response to bacterial flagellin and LPS/lipoproteins, respectively. Duplication events to the zebrafish genome resulted in two copies of TLR4, tlr4a and tlr4b. LPS stimulation in the zebrafish was not recognized by tlr4, indicating a tlr4 independent pathway for LPS in zebrafish. Other TLRs have shown
conservation between humans and lower level vertebrates. TLR5, is conserved in vertebrates from fish to human. TLR5 also underwent a duplication event in the zebrafish, yielding tlr5a and tlr5b. TLR9 also appears to have conservation with zebrafish thr9 (Luc, unpublished). 

P. aeruginosa has evolved ways to evade the immune response, treatments, and increase its virulence through various virulence factors in addition to LPS.

P. aeruginosa, like other Gram-negative bacteria, utilizes secretion systems to disseminate proteins and other toxins to enhance virulence. P. aeruginosa uses two types of secretion systems type II and type III, denoted T2SS and T3SS. T2SS utilizes Xcp and Hxc to mediate secretion of proteases, lipase, and toxins into the extracellular medium or onto the surface of the bacterium. T3SS uses a needle complex and translocation apparatus to secrete proteins such as exoenzymes (ExoU, ExoT, and ExoS) directly into the eukaryotic host cell. These proteins help the bacterium inhibit inflammasome activity, and target host proteins involved in phagocytosis, endocytic trafficking and immune cell migration. Additionally, this catalase-positive bacterium has the ability to neutralize hydrogen peroxide, an otherwise toxic ROS produced by professional phagocytes. The arsenal of virulence factors of P. aeruginosa makes this bacterium very difficult to treat, leading to persistent and recurring infections, especially in CF patients.

CF is a congenital disease that is caused by malfunction of chloride channels encoded by the cystic fibrosis transmembrane conductance regulator gene (CFTR). This disease is most commonly associated with the lungs; however, it is a multi-organ disease affecting the pancreas, small intestine, and the liver. Despite extensive antibiotic therapy, CF patients are prone to persistent P. aeruginosa infections. The low membrane permeability of P. aeruginosa bacteria coupled with the presence of an efflux pump increases antibiotic resistance and makes this...
bacterium difficult to treat. P. aeruginosa persists in these patients by forming biofilms, which are produced through the release of exopolysaccharides, which enable microcolonies to attach to surfaces and encase in a biopolymer matrix. Biofilm growth protects P. aeruginosa from destructive immune components. Colonization of P. aeruginosa coupled with biofilm production and an increase in antibiotic resistance enhances the morbidity and mortality of CF patients with persistent infections.

The prevalence of P. aeruginosa infections in nosocomial settings and with CF patients has led to the extensive investigation of pathogenesis. However, increased evidence of antibiotic resistance and immune evasion warrants investigation of the host response. Understanding of the host response, including gene regulation, upon bacterial infection could potentially lead to gene-targeted therapeutics. This paper will show a potential functional role for miR-199 during bacterial infections using the zebrafish model.

**Zebrafish as a model organism**

Animals are commonly used to study basic biological processes and to model human disease states. The zebrafish has become a valuable vertebrate model for basic and translational research. Since the 1970s, beginning with the work of Dr. George Streisinger, the zebrafish model has been used to explore cardiovascular, muscle, toxicant, and pathogen studies. The zebrafish model system has many benefits, which include ex utero fertilization, fast generation time, large sample sizes, optically clear embryos, an annotated genome, and amenability to genetic manipulation. As a result of the zebrafish sequencing project, we now know that there are many genetic similarities between zebrafish and humans, making zebrafish an attractive model for use in the investigation of biomedical science research. In annotating the zebrafish genome, researchers found 70% of protein-coding human genes were related to genes in the zebrafish. Further, it
was found that 84% of human genes associated with disease had a zebrafish counterpart\textsuperscript{59}. With such high genomic conservation, particularly in relation to genes associated with disease, zebrafish have become a useful model in which to explore questions related to human health. Advantages of the zebrafish model system, as well as biomolecular tools, have given researchers the ability to make observations and answer questions using zebrafish that could not be answered in other model systems.

The genetic amenability of the zebrafish has allowed for the generation of transgenic and mutant zebrafish lines as well as the evaluation of specific gene or miR function by using morpholino oligonucleotide (MO) knockdown applications. Transgenic reporter lines, such as Tg(\textit{mpx:EGFP}) and Tg(\textit{mpeg1:mcherry}), use gene promoters to drive the expression of fluorescent proteins and aid in study of the innate immune system by allowing for \textit{in vivo} visualization of neutrophils and macrophages, respectively. Functional roles for specific gene products can be determined in zebrafish by employing tools such as MOs to knockdown expression of genes of interest\textsuperscript{60}. MOs accomplish targeted gene “knockdown” via sterically blocking the region of interest and preventing either protein synthesis or messenger RNA (mRNA) splicing of transcripts. MOs can be used to prevent the synthesis of mature miRs by binding to the particular transcript of interest within the pri-miR complex and my also binding the cleavage sites for either Drosha or Dicer.

One unique hallmark of the zebrafish model in the context of disease is the temporal separation of their innate and adaptive immune systems. Adaptive immune markers are not present until 4 days post fertilization (dpf) and a fully functioning adaptive immune system is not observed until 4-6 weeks post fertilization\textsuperscript{61}. This characteristic of the zebrafish allows for the direct observation of the innate branch of the immune system in zebrafish embryos and larvae.
The isolation of the innate immune system gives researchers a model to study the first line of defense, without the complication of adaptive immunity. Key components of the innate immune system, such as phagocytes, complement factors, and anti-microbial peptides, are all present in the zebrafish embryo during early development\textsuperscript{62,63}. Phagocytes are present in the zebrafish by 48 hpf, with macrophages differentiating from HSC first at 18 hpf, followed by neutrophils between 30 hpf\textsuperscript{64,65}. Zebrafish respond to microbes in a similar fashion to the mammalian model with recognition through PAMP-PRR interactions. Many signaling transduction components of the mammalian TLR pathways are present in the zebrafish, including putative mammalian orthologs for the TLR receptors and their cytoplasmic adaptor proteins, such as Myd88, TIR-domain-containing adapter-inducing interferon-β (TRIF)\textsuperscript{74}.

The above studies are examples of how the zebrafish model has gained ground as a model for inflammatory and infection states. Many other research studies have proven zebrafish to be an ideal model for infectious disease\textsuperscript{79–83}. Additionally, the optical clarity of zebrafish embryo has enabled the observation of host interaction with infectious disease in real time, including chemotaxis, phagocytosis, and pathogenesis\textsuperscript{84}. The zebrafish has been used to model viral, fungal, and bacterial infections. To date the zebrafish has served as a disease model for bacterial infections such as \textit{Edwardsiella tarda}, \textit{Mycobacterium haemophilum}, \textit{Salmonella typhimurium}, \textit{Staphylococcus aureus}, and \textit{Pseudomonas aeruginosa} (\textit{P. aeruginosa})\textsuperscript{79–83}.

Immersion, duct of Cuvier (DC) injections, and tail vein injections can all cause a systemic infection in zebrafish, whereas, hindbrain injections into closed ventricles result in a localized infection\textsuperscript{84}. Clatworthy et al. demonstrated that injection of \textit{P. aeruginosa} into zebrafish circulation leads to a lethal infection\textsuperscript{83}. The establishment of a bacterial model for \textit{P.
aeruginosa in the zebrafish allows for the study of bacterial pathogenesis in the context of a human disease Cystic Fibrosis (CF) and the innate immune response.

**MicroRNAs**

MicroRNAs (miRs) are short, endogenously expressed; non-coding single-stranded RNA (ssRNA) fragments, typically 22-24 nucleotides in length. MiRs were first discovered in 1993 by Victor Ambros and colleagues Rosalind Lee and Rhonda Feinbaum, during heterochronic studies in *Caenorhabditis elegans* (*C. elegans*). Lin-4 was discovered to have an essential role in normal temporal *C. elegans* development; however, lin-4 is non-protein coding, a phenomenon determined through site-directed mutagenesis. The sole function of Lin-4 expression is to negatively regulate lin-14, a protein-coding gene whose timed expression is also vital in nematode development. Sequence complementarity between lin-4 and the 3’ untranslated region (UTR) of lin-14 showed RNA-RNA interaction leading to decreased lin-14 protein expression, while lin-14 transcript levels remained the same. The discovery of non-coding RNA fragments that regulate gene expression post-transcriptionally, were originally thought to be unique to *C. elegans*. The presence of these small non-coding RNAs, later coined miRs, have now been found in a vast variety of entities from low level organisms, such as sponges, to high level vertebrates and humans.

**MiR Biogenesis**

MiRs are well-conserved amongst different organisms, which include worms, flies, and mammals. The biogenesis of miRs follow a similar canonical pathway in most species beginning in the nucleus with a long primary miR transcript (pri-miR) transcribed with the help of RNA polymerase II (Bartel et al). The transcription of pri-miR stems from intergenic, intronic, and exonic regions of the genome. The biogenesis of mature miRs is initiated by Drosha, an
RNase III endonuclease, which recognizes tertiary structure, as opposed to sequence motifs, and cleaves pri-miR into a shorter stem-loop precursor ∼70 nucleotides, known as pre-miR\textsuperscript{22,23}. Drosha, a large 160kDa protein, contains two RNase III domains and a double stranded RNase binding domain (dsRBD) needed for proper processing\textsuperscript{24}. This RNase III endonuclease is usually accompanied by a cofactor known as DiGeorge syndrome critical region gene 8 (DGCR8) in vertebrates or Pasha in invertebrates to form a microprocessing complex\textsuperscript{22–24}. The biochemical role of this cofactor is not fully understood; however, it is believed to aide Drosha in substrate recognition. Drosha cleavage results in pre-miR with a ∼22 nucleotide stem, a terminal loop, and a 3’ overhang of ∼2 nucleotides. This structure was shown by Cullen et al to be an important pre-miR structural requirement\textsuperscript{25}. Following nuclear processing the intermediate product of Drosha, pre-miR, is then exported via Ran-GTP dependent Exportin 5\textsuperscript{26}. Once in the cytoplasm pre-miR is subjected to additional cleavage via another RNase III endonuclease, Dicer\textsuperscript{23}. Dicer completes cytoplasm cleavage of pre-miR into a mature miR duplex. The long N-terminal segment of Dicer contains a PAZ (Piwi Argonaut and Zwille) domain. This PAZ domain can also be found in a highly conserved group of proteins known as argonaute proteins (Ago), which bind to the 3’ protruding end of the stem-loop structure created by Drosha cleavage in the nucleus\textsuperscript{22}. Ago proteins do not appear to be required for Dicer cleavage; however, their role may be in providing stability via effector complex formation. Dicer cleaves pre-miR hairpin structures, forming a miR-duplex containing 5 and 3 prime arms, denoted 5p and 3p. Helicases present in the cytoplasm unwinds the miR-duplex structure separating the 5p and 3p arms. The miR arm with the higher thermodynamic instability at the 5’ end of the fragment is typically incorporated into the RNA inducible silencing complex (RISC) with the help of Ago, forming an effector complex\textsuperscript{22,27}. The other strand of the duplex is normally degraded, as it is not stabilized.
with RISC effector complex incorporation. In some cases both arms of the duplex are expressed or a different arm is incorporated depending on tissue specificity, a phenomenon known as “arm switching”\textsuperscript{28,29}. The mature miR and effector complex regulate genes post-transcriptionally through RNA-RNA interactions in 3’ UTR of gene transcripts\textsuperscript{10}. Although miRs are 22-24 nucleotides in length their RNA-RNA interactions post-transcriptionally are not solely dependent on complete complementarity (Figure 1).

Figure 2. The biogenesis of miRs. Modified from Szabo et al. 2010.
Targeting of MiRs

Typically 6-8 nucleotides within the miR fragment, exhibiting Watson-Crick pairing is sufficient to target mRNA\(^1\). This region of RNA-RNA complementarity is known as the miR seed region. The “seed” region located at the 5’ end of the miR is centered on nucleotides 2-7. MiR seed sites can be classified as 8mer-1A, 7mer-m8, or 7mer-1A in order of hierarchy depending on complementarity in the 2-7 seed region. The 8 mer-1A site is an exact match to nucleotide 2-8 followed by an adenine “A” in position 9, 7-mer-m8 is an exact match to 2-8nt, and 7mer-1A is an exact match to 2-7 followed by an adenine.

The binding of miR to target sites is also influenced by other factors in addition to seed region complementarity, which include positioning within the 3’UTR; the location of stop codons; spacing between miR binding sites; and the secondary structure of target mRNA\(^30,31\). Base-pair complementarity of the complete miR:mRNA interaction increases the likelihood of endonucleolytic cleavage leading to mRNA degradation. Endonucleolytic cleavage of mRNA is tolerable to few mismatch base-pairings; however, even in the presence of perfect Watson-Crick pairing miR processing machinery such as Dicer, Ago, RISC, and the interaction of other proteins may be required for successful degradation\(^32-34\).

The binding of miRs within the 3’UTR region of target genes may also prevent gene expression through inhibition of proper translation. Cytoplasmic mRNA transcripts are translated into protein via ribosome initiation, elongation, and termination. Studies show some miR targeted mRNA remain as polysomes, which suggests that translational repression of miR occurs after ribosome initiation\(^31\). The interaction of miR:mRNA can also occur at the 5’UTR of target genes showing repression and activation results\(^35\). The mechanism of miR activation remains
poorly understood; however, it appears to be mediated by Ago proteins\textsuperscript{35}. The large scope of possibilities that influence miR:mRNA interactions allow for specific control of gene regulation.

Imperfect binding at the 3’ UTR of gene transcripts allows for one single miR to bind and regulate the expression of hundreds to thousands of gene products. MiRs also have the ability to bind a single transcript more than once and a single transcript can be targeted by multiple miRs\textsuperscript{36,37}. MiRs that bind target sites with complete complementarity or base pairing within the seed region improve the likelihood of bona fide targets. Genome-wide computational searches, such as Targetscan, have developed a method using experimental requirements of bona fide miR:mRNA interactions; taking into account the complexity of miR binding and conservation of sites to allow for algorithm target predictions\textsuperscript{20,22,38–40}. These resources allow for identification of putative targets and target sites for a given miR or gene input. Genome wide computational searches have enabled the identification of 1881 miRs in the human genome, a drastic increase since their discovery in 1993\textsuperscript{20,41–44}.

**MiRs in Immunity**

The 1881 miRs identified in the human genome are believed to regulate upwards of 60-80% of human gene expression\textsuperscript{20,41}. Precise control of gene expression is vital for of homeostasis throughout the body. Physiological and pathological issues can arise in the absence or inability of a system to return to homeostasis after disruption. The discovery of miRs introduces a new class of regulators that may help answer underlying questions regarding the regulation of genes involved in disease progression, immunity, and other cellular processes. Expression profiles of miRs have been described in a wide range of tissues and aberrant miR expression profiles have been associated with at least 70 diseases (Table 1)\textsuperscript{39,45}. 
<table>
<thead>
<tr>
<th>Disease/Inflammation Type</th>
<th>miR</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac hypertrophy</td>
<td>miR-23a, miR-23b, miR-24, miR-195, miR-199a, and miR-214</td>
<td>11</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>miR-155, miR-146</td>
<td>12</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>miR-510</td>
<td></td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>miR-146a</td>
<td></td>
</tr>
<tr>
<td>Bacterial infection</td>
<td>miR-155</td>
<td></td>
</tr>
<tr>
<td>Myeloproliferative disorders</td>
<td>miR-125b, miR-155, miR-146a</td>
<td></td>
</tr>
<tr>
<td>Alzheimers</td>
<td>miR-9, miR-128a, miR-125b</td>
<td>13</td>
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Whether miRs functioned within the immune system was examined in experiments where miR biogenesis was altered. The conditional deletion of Dicer, a cytoplasmic endonuclease essential for the production of mature miRs, prevents hematopoietic stem cells (HSCs) from reconstitution in the mouse model\(^46\). HSCs are typically able to renew themselves and give rise to new blood cells, including immune progenitor cells needed for the establishment of both innate and adaptive immune cells. The deletion of dicer in T and B-lymphocytes led to fewer T-cells in the thymus and periphery, while also inhibiting B-cell development\(^47\). Furthermore, knockout of Ago2, the protein responsible for stabilizing the RISC complex in HSCs, impaired B-cell and erythroid differentiation\(^48\). The inhibition of miRs at the level of biogenesis drastically impacted immune cell progenitors, indicating a role for miRs in the immune system.

An ongoing task is now identifying the functional roles of individual miRs. The use of microarrays, deep sequencing, and other bioinformatics approaches has led to key findings regarding miR expression within the immune system. As of 2012, upwards of 100 miRs were found to be expressed in cells destined for the immune response\(^49\). Aberrant miR expression profiles in various malignancies and infection states have indicated a potential role for individual
miRs in mammalian immunity. miR-155 and miR-146 have been extensively studied and have both shown important roles in the innate immune system.

Profiled as a typical immune response regulator, miR-155 is a common target of broad range inflammatory mediators such as TNF and interferon (IFN) \(^{48}\). MiR-155 expression is upregulated in monocytes, macrophage, and myeloid dendritic cells upon stimulation with LPS, IFNβ, and polyriboinosinic: polyriboctydyllic acid (Poly(I:C)) \(^{48,50–52}\). MiR-155 upregulation is initiated through the recognition of bacterial or viral PAMPs by TLRs and involves NF-κB and Jun N-terminal kinase (JNK) pathways in macrophage cells. Blocking the JNK pathway with chemical inhibitors prevented the upregulation of miR-155 following inflammation in murine macrophages\(^{50}\). O’Connell et al. identified Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1) as a direct 3’UTR target of miR-155 in the hematopoietic system\(^{53}\). Either SHIP1-deficiency or miR-155 overexpression in HSCs led to myeloproliferative phenotypes. The specific function of miR-155 in macrophage and HSCs still remains unclear. However, miR-155 has an apparent role in myeloid and lymphoid development as evidenced by the association between overexpression of miR-155 and myeloid proliferative disorders, such as myeloid leukemia \(^{53}\). Dendritic cells deficient in miR-155 have an impaired ability to trigger T-cell activation following antigen presentation; suggesting miR-155 may act as a “bridge” between the innate and adaptive immune systems\(^{54}\).

A role for miR-146 in the development and homeostatic control of myeloid and lymphoid cells has also been documented. MiR-146 was one of the first miRs to be identified in regulating the mammalian response to infection. MiR-146 is upregulated in immune cells upon stimulation with bacterial components such as LPS, as well as IL1 and TNF\(^{52,55}\). Bacterial infection in zebrafish induces miR-146 expression in a myeloid differentiation primary response gene 88
(MyD88)- TNF Receptor-Associated Factor 6 (TRAF6) dependent manner. Targets of miR-146 include Interleukin-1 receptor-associated kinase (IRAK) and TRAF6, which play key roles in TLR and IL-1 receptor signaling pathways during innate immunity\textsuperscript{48}. The downregulation of these pathways in the presence of miR-146 indicates that miR-146 is a negative regulator of inflammation. Human monocytes expressing miR-146 attenuate the expression of TNF, IL-6 and IL-12\textsuperscript{55}.

MiR-155 and miR-146 were originally identified as inflammation responsive miRs as both were upregulated by NF-κB. Upon further investigation, functional roles for each of these miRs were found within different facets of the innate immune system\textsuperscript{48}. It is therefore likely that other miRs that regulate or respond to NF-κB will also play roles in the innate immune response. NF-κB is a transcription factor that induces the expression of many proinflammatory cytokines and thus contributes to the innate immune response as well as many inflammatory disorders. The NF-κB pathway is constitutively active, with high activity of IKKβ (an activator of NF-κB), in many cancers including breast cancer, melanoma, pancreatic cancer, and acute myeloid leukemia\textsuperscript{14}. Inhibition of IKKβ prevents the translocation of NF-κB to the nucleus, thereby suppressing the transcription of proinflammatory cytokines. IKKβ, is a direct target of miR-199a in human epithelial ovarian cancer cells\textsuperscript{14}, indicating a role for miR-199 in the NF-κB pathway and potentially the innate immune response. The functional role of miR-199 in the innate immune system has not yet been investigated in vivo.

**MiR-199 Family**

In humans, the miR-199 family consists of three members: *Homo sapiens* (hsa)- miR-199a-1, miR-199a-2, and miR-199b; which yield mature sequences hsa- miR-199a-5p, hsa- miR-199a-3p, and hsa- miR-199b-5p\textsuperscript{42,44}. In zebrafish, miR-199 also has three family members:
*danio rerio* (dre)- miR-199-1, dre-miR-199-2, and dre-miR-199-3, yielding mature sequences dre- miR-199-5p, dre- miR-199-3p, and dre- miR-199-3-3p\(^{42-44}\). The function of miR-199 has been studied in humans, mice, rats, and zebrafish. Many studies primarily focused on hsa-miR-199a-5p, because previous literature suggested miR-199a-3p was degraded. However, microarrays have detected high expression of both miR-199a-5p and miR-199a-3p in various cell and tissue types, suggesting both mature sequences may play important functional roles. The extensive use of microarrays has shown aberrant expression of miR-199a in animal models of heart failure, lung fibrosis, cancer, and liver disease as well as expression during skeletal development, and the maintenance of mesenchymal stem cells\(^{14,56-59}\).

**Functional Roles for miR-199 in Human Health**

Animal models of heart disease have revealed a role for miR-199a-5p in cardiac myocytes, cardiac hypertrophy, and end stage heart failure\(^{59-61}\). Hsa-miR-199a-5p is downregulated during hypoxia preconditioning and early ischemia in cardiac myocytes\(^{60}\). Reduction of hsa-miR-199a-5p is required for the expression of hypoxia inducible factor 1 (HIF-1\(\alpha\)). Hsa-miR-199a-5p knockdown during normoxia led to an upregulation of HIF-1\(\alpha\). Altogether, these data show that HIF-1\(\alpha\) is a direct target of miR-199. Downregulation of miR-199a-5p prior to hypoxia increased inducible nitric oxide synthases, (iNOS) production. This increase in iNOS was dependent upon the downregulation of miR-199a-5p and increased HIF-1\(\alpha\) activity. Downregulation of hsa-miR-199a-5p reproduces a similar expression profile to that of hypoxia preconditioning, which could lead to protection against future hypoxic damage\(^{60}\).

Significant upregulation of *Rattus norvegicus* (rno)-miR-199a-5p was found during cardiac hypertrophy. Rno-miR-199a-5p function contributes to the maintenance of
cardiomyocyte cell size and regulation of cardiac hypertrophy. HIF-1α is a potential regulator in cardiomyocyte development and is significantly downregulated with the overexpression of rno-miR-199a-5p. End stage failing adult human hearts and hypertrophic mouse hearts also displayed an overexpression of hsa-miR-199a-5p. Overexpression of hsa-miR-199a phenotypically alters cardiomyocytes to elongate and serially assemble. MiR-199a can regulate signaling pathways by targeting transcription factors, such as NF-κB and Hif-1α, which influence proinflammatory cytokines and cell maintenance.

MiR-199a can also be regulated by signaling pathways such as transforming growth factor (TGF-β), a cytokine which plays a role in immunity and is known to induce the expression of miR-199a in fibrosis lung, kidney, and liver fibrosis models. Inhibition of Caveolin 1 (CAV1), a target of miR-199-5p, altered the ability to endocytosis TGF-β receptors, promoting TGF-β signaling. MiR-199a-5p overexpression in lung fibroblasts enhances the ability of fibroblasts to migrate, proliferate, and differentiate into myofibroblasts. Fibrosis is a typical result of inflammation in this case, perturbed by overexpression of miR-199.

Over expression of proinflammatory cytokines is a hallmark of cancer development. MiR-199a has been shown to play a role in proinflammatory cytokine expression and the development of ovarian cancer. Epithelial ovarian cancer (EOC) cells extracted from humans showed the dyregulation of hsa-miR-199a affected tumor progression, production of proinflammatory cytokines, and cell response to chemotherapy treatments such as cisplatin. Gene expression profiles of ovarian cancer cells with different outcomes revealed IKKβ and rapamycin (mTOR) as potential targets of hsa-miR-199a. Hsa-miR-199a could act as a gene marker for ovarian cancers and help direct treatment approaches.
Microarray studies looking at various conditions such as *Schistosomiasis japonica* infection in murine liver (45 dpi), aging Mesenchymal stem cells (MSCs) in rhesus monkeys, and the effects of perfluorooalkyl chemicals (PFCs) in human subjects, all revealed dysregulation of miR-199. MiR-199-5p and 3p were both increased in *Schistosomiasis japonica*-infected murine livers, miR-199a-3p expression was decreased in aging MSC of rhesus monkeys, and serum levels of miR-199-3p were increased in humans exposed to PFCs. Ex vivo studies have implicated a role for miR199 in human disease and within immune pathways such as NF-κB signaling. Despite numerous functional roles for miR-199 family members in animal health and inflammatory signaling, the role of miR-199 in innate immunity in vivo has yet to be investigated in the context of innate immunity in vivo.

**Materials and Methods:**

**Zebrafish Maintenance**

Adult zebrafish were maintained at 28 °C on a 16 hour light/ 8 hour dark cycle in the Zebrafish Facility at the University of Maine in Orono. Embryos were obtained from natural spawnings of Adult AB strain, *Tg(mpx:GFP)* and *Tg(mpeg1:mCherry)*. Fertilized embryos were collected and placed in 50 ml of egg water (60 mg Instant Ocean sea salts/L) at 28°C incubation. New egg water was administered every 24 hours and non-surviving embryos were removed. For visualization purposes some embryos were manually dechorionated at 24 hours post fertilization (hpf) and subjected to egg water containing 1-phenyl-2-thiourea (PTU) (1mg/ml) to prevent pigmentation. Embryo care and maintenance followed the guidelines set forth by the Institutional Animal Care and Use Committee at the University of Maine.
Microinjection of Morpholino Oligonucleotide

Anti-sense microRNA morpholino oligonucleotides (MO) were designed using sequence information from the Sanger Institute's miRBase (Accession # MIMAT0003155) and synthesized by Gene Tools, LLC (Eugene, OR). Oligonucleotides were diluted in 1× Danio buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5.0 mM HEPES; pH 7.6) and 0.5% phenol red indicator (Sigma-Aldrich, St. Louis, MO). MOs were designed to the star strand and the dicer cleavage site on the star strand. MiR-199-3p 5’-TCTAACCAATGTGCAGACTACTGTA 3’-Fluorescein and MiR-199-1-3p 5’-TCTAACCAATGTGCAGACTACTGTA 3’ were complemented with standard MO 5’-CCTCTTACCTCAGTTACAATTTATA-3’. Fertilized embryos were injected in the yolk with approximately 2 nl (12 ng/nl) at the 1–2 cell embryonic stage and injected at 40–50 psi.

Bacterial injections

*Pseudomonas aeruginosa* infections were performed with *Pa* (*p67t1*), which constitutively expresses the fluorescent protein dTomato. *P. aeruginosa* (*p67t1*) was incubated overnight in Luria Broth (LB), 750 µg/ml ampicillin. Cultures were spun at 2450 x g for 5 minutes at 4°C. Pellets were resuspended and washed a total of three times in sterile 1X Dulbecco's phosphate buffered saline (DPBS) (Life Technologies). The optical density (A₆₀₀) was measured and adjusted to 1.8 - 2 x 10⁷CFU/ml. *P. aeruginosa* bacteria were prepared in a 0.5% phenol red injection solution and diluted with DPBS. Zebrafish embryos were anesthetized in a 4 mg/ml tricaine solution (Western Chemical, Ferndale, WA) prior to infection at 48hpf. Fish were mock-infected with DPBS containing 0.5% phenol red. Fish were injected via the cardinal vein with 1.5 nl of solution for systemic evaluation and in the hindbrain ventricle for
localized infection. Injection volume of 1.5 nl was diluted in PBS and plated onto LB plates before and after bacterial injections to determine inoculum (CFU/ml). Fish injections were rotated between control and morphants to minimize change in inoculum size.

**Mortality**

Zebrafish were subjected to four conditions, with 20 - 60 zebrafish embryos per condition. The conditions included: PBS Control MO, PBS miR-199 MO, *P. aeruginosa* infected Control MO, and *P. aeruginosa* infected miR-199 MO. The fish were monitored for 5 days post infection (dpi) for survival. Statistical analysis and the production of mortality curves were completed using the Mantel-Cox test with Prism 6 software.

**Bacterial Burden Assay**

Embryos were washed in 4 mg/ml tricaine solution 6 hours post infection (hpi). Single fish were then homogenized in 200 µl of PBS along with two 4.2 mm metal beads using a Bullet Blender at speed 8 for 2 minutes (Next Advance). Homogenates were diluted in PBS and plated onto Cetrimide agar to isolate *P. aeruginosa* colonies. Plates were incubated for 16 hours at 37 °C.

**In Situ Hybridization**

AB strain embryos were dechorionated 24hpf and treated with PTU (1 mg/ml). Embryos were fixed at 54 hpf with 4 % (wt/vol) paraformaldehyde (PFA) and washed in 1x PBS-Tween solution before being stored at 4 °C. For miRNA *in situ*, three washes of 1-methylimidazole buffer (1-MIB) followed by treatment with 0.16M 1-ethyl-3-((3-dimethylaminopropyl) carbodiimide (EDC) were completed prior to dehydration steps (Sigma-Aldrich, St. Louis, MO). Embryos were washed 5 x 5 min with PBS-Tween and dehydrated 1 x 5 min with the
following solutions: 33% MeOH / 66% PBS-Tween, 66% MeOH / 33% PBS-Tween, and 100% MeOH before being stored for 48 hrs at -20 °C. Dehydrated embryos were treated with Proteinase K (Fisher) for 15 to 45 minutes and then fixed (20 minutes) in 4% PFA and washed 5 × 5 min PBS-Tween. Embryos were incubated in prehybridization solution (50% Formamide, 5 × sodium chloride-sodium citrate buffer, 50 µg/mL heparin, 500 µg/mL yeast tRNA, 0.1% Tween20, pH of 6.0 with citric acid) for 4 hours in 65°C water bath, and then hybridized overnight at 65°C with a fluorescein-labeled MO probe:

MiR-199-3p 5’-TCTAACCAATGTGCAGACTACTGTA 3’

Embryos were washed in PBS-Tween, blocking buffer, then incubated in either anti-digoxigenin Ab or anti-fluorescence Ab (Roche) for 2 hours at RT. Embryos were then subjected to coloration buffer and developed in a 0.45% NBT / 0.35% BCIP (Roche) coloration buffer solution until desired color was achieved and fixed in 4% PFA. Imaging was completed using a Zeiss Axio Imager Z1 microscope with a Zeiss ApoTome attachment.

**RNA extraction and cDNA synthesis**

Ten zebrafish were collected from treatment at 1, 3 and 6 hpi. TRIzol RNA extraction procedure was completed according to the manufacturer’s protocol. The extracted RNA was then analyzed for concentration using NanoDrop Ultra Violet spectroscopy. RNA template was diluted in nuclease-free water (NFW) to yield a RNA concentration of 0.5-1 µg/mL. Quanta miRNA cDNA synthesis kit was followed according to the manufacturer’s instructions. The reactions were then diluted 1:20 in 180 µL NFW for a final volume of 200µL cDNA.

**Quantitative PCR (qPCR)**

Quantitative PCR was performed using PerfeCTa® SYBR® Green FastMix per the
manufacturers protocol (Quanta). Ct values were normalized to RNU6. Cycling conditions were: 95°C (3:00 min) followed by 40 cycles of 95 °C (5 sec), 55°C (sec), 72°C (7 sec). A melting curve was generated to indicate specific product production. Primers used were as follows; MiR-199 primer (hsa-miR-199a-3p: ACAGUAGUCUGCACAUAUGGUUA) IDT®, PerfeCTa® Universal PCR Primer, RNU6-1 (GUGCUCGUUCGGCAAGCACAUAUCUAAAUAUGGAACGAUACAGAGAA GAUUAGCAUGGCCUCUGCAGCAAGAUGCAUGACACGCAAAUUGGUGAAGCGU UCCAUAUUUU) Quanta® and Lyn (5’-CGAAAGCTGGATAAAGCATGCG 3’) (5’-CTGCTCTCAGGTCTCGGTGG 3’) Quanta®. At least three biological replicates were used per time point. Data were analyzed using the comparative Ct method.87

**Respiratory Burst Assay**

A working solution of dihydrodichlorofluorescein (H2DCFDA) was prepared by combining, in a 1:1 ratio, H2DCFDA stock solution (1mg/mL in DMSO) with DMSO to a final concentration of 500µg/mL. A working solution of PMA was prepared in a 1:49 ratio of PMA to NFW for a final concentration of 20µg/mL. A dosing solution of H2DCFDA was prepared to fill 48 wells with a final concentration of 1µg/mL of H2DCFDA in egg water. Another dosing solution of H2DCFDA + PMA was prepared to a final concentration of 400nL/mL PMA and 1µg/mL H2DCFDA to fill the remaining 48 wells. All 96 wells were used and split into four conditions: control, miR-199 knockdown (KD), control + PMA, and miR-199 + PMA, using 1 embryo per well. H2DCFDA in egg water dosing solution (100µL) was added to the first 48 wells. To the remaining 48 wells, 100µL H2DCFDA + PMA solution was added. The plate was then covered with aluminum foil and incubated for 3 hrs at 28°C. Samples were read using a fluorescence microplate reader.88,89
Results:

miR-199 is dysregulated within the zebrafish upon *P. aeruginosa* infection

Following RNAseq analysis, miR-199 was found to be upregulated in the zebrafish upon *P. aeruginosa* infection and CFTR knockdown (Figure 1). This result identified miR-199 as a miR that may be implicated in the innate immune response, as well CF. However, further analysis was necessary to confirm this preliminary hypothesis.

![Figure 3](image.png)

Figure 3. RNAseq analysis identifies miR-199 as upregulated upon *P. aeruginosa* infection. Zebrafish were subjected to *P. aeruginosa* via duct of cuvier (DC) injection. RNA was isolated
at 6 hours post infection (hpi). MiRs were binned based on high expression (> 10 log (counts per million)) and high fold change (> log 2).

In order to confirm that *P. aeruginosa* was indeed influencing the expression of miR-199, we measured miR-199 expression as a result of *P. aeruginosa* infection alone, without the influence of CFTR knockdown. It was shown that miR-199 expression was significantly downregulated at 1hpi and significantly upregulated at 6 hpi (Fig. 4). The significant upregulation in response to bacterial infection at 6hpi further suggested a potential functional role for miR-199 in the innate immune system.

![Figure 4](image.png)

**Figure 4.** miR-199 expression increases 2.4-fold upon *P. aeruginosa* infection. 2 days post fertilization (dpf) zebrafish embryos were mock-infected with PBS or infected with *P. aeruginosa* (p67T1) through tail vein injections to mimic systemic infection. RNA was isolated and converted to cDNA at 1, 3, and 6 hpi. qPCR was performed to analyze the expression of miR-199 upon bacterial infection.
MiR-199 expression is localized to the inner ear, heart, and anterior kidney

Previous studies have shown miR-199 expression in murine inner ear structures as well as murine and zebrafish hearts, specifically in cardiomyocytes, aortic/cardiac valves, and endocardial cells\textsuperscript{52–54,90}. To observe the spatial and temporal expression of miR-199 in the developing zebrafish embryo before proceeding with function studies we performed \textit{in situ} hybridization (ISH). As expected, due to the conserved nature of mature miRNA transcripts, miR-199 expression was highly expressed in the ear and heart regions throughout the time course, as indicated in purple (Figure 3). At 48 hpf the yolk syncytial layer (YSL) surrounding the yolk sac of the embryo displays high miR-199 expression. MiR-199 is also expressed in the anterior kidney, a region of hematopoetic stem cell origination, similar to the bone marrow in humans.

\textbf{Figure 5.} \textit{In situ} hybridization indicates that miR-199 expression is localized to the ear, heart and anterior kidney of the zebrafish. PTU treated embryos were fixed at 24, 48, 72, and 96 hpf. Whole mount ISH was performed for the detection of mature miR-199 transcripts.
Susceptibility to Bacterial Infection Decreases upon Loss of MiR-199

MiR-199 is upregulated during systemic infections with *P. aeruginosa* and expressed in the anterior kidney of the zebrafish. We hypothesize a functional role for miR-199 in the context of innate immunity and implemented a reverse genetic approach to knockdown mature miR-199 transcripts via miR-199 MO injection at the single cell stage of zebrafish development. Upon knockdown of miR-199, *P. aeruginosa* infected zebrafish showed a decreased bacterial burden at 6hpi as well as an increased survival rate over the course of a 5-day infection. Over the 5-day survival observation there was no significant decrease in survival between the control and miR-199 MO mock infected populations. However, miR-199 morphants displayed roughly a 50% increase in survival upon systemic *P. aeruginosa* infection compared to the controls (Figure 4).

Figure 6. Knockdown of miR-199 via oligonucleotide morpholino decreases bacterial burden and increases overall survival of zebrafish systemically infected with *P. aeruginosa.*

A.) Embryos were washed in 4 mg/ml tricaine solution 6 hpi. Single fish were then homogenized in 200 µl of PBS along with two 4.2 mm metal beads using a Bullet Blender at speed 8 for 2 minutes (Next Advance). Homogenates were diluted in PBS and plated onto Cetrimide agar to
isolate *P. aeruginosa* colonies. Plates were incubated for 16 hours at 37 °C. B.) Zebrafish were injected at the 1–2 cell stage with miR-199 MO or control MO. 48 hour post fertilization (hpf) the fish were subjected to bacterial infection. Control and miR-199 morphants were mock-infected with PBS or infected with *P. aeruginosa* dTomato via tail vein injections. Embryos were screened using the Zeiss microscope to ensure systemic *P. aeruginosa* infection. Embryo survival was observed for 5 dpi. (Statistical analysis was determined using a Log-rank test (**** p<.0001, *** p<.001, ** p<.01).

The Src Family Kinase (SFK) *Lyn* is a putative target of miR-199

A target scan was performed and SFK *Lyn* was identified as a potential target of miR-199. Given the implications of *Lyn* in multiple biological pathways including those within the immune system, we performed a miR-199 KD and observed changes in *Lyn* expression. Upon miR-199 KD *Lyn* expression increased roughly 5-fold compared to the control.

![Relative Expression](image.png)

**Figure 7. Src Family Kinase (SFK) *Lyn* is a putative target of miR-199.** Zebrafish embryos at the one cell stage of development were injected with control and miR-199 MO. RNA was isolated at 1 day post injection and converted into cDNA. qPCR was performed using primers specific to the zebrafish *Lyn* gene.
**Discussion:**

Human health is continually being complicated by microbial antibiotic resistance and compromised host immunity. Gaining a better understanding of host immunity and gene regulation will help direct therapeutics and strategies to enhance the immune response. Targeting host genes avoids the selective pressures faced by therapeutics, such as antibiotics, that are directed against specific infectious agents. Profiling of aberrant gene expression during infections has the potential to identify unique functional roles for genes within the immune system. MiR research has provided insight into gene regulation mechanisms and may further help advance human therapeutic design. In this study, we seek to understand the role that miRs play on gene regulation in the context of the innate immune response.

As part of a large collaborative Centers of Biomedical Research Excellence (COBRE) project, multiple institutes sought to “Determine the effects of very low concentrations of Arsenic (As) on Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) expression and the innate immune response using a toxicogenomic approach”. There is evidence that CFTR is linked to the innate immune response, as mutations in CFTR result in increased susceptibility to lung infection by *P. aeruginosa* in humans. The zebrafish has been shown to be a useful model to study the host innate immune response and the pathogenesis of *P. aeruginosa* associated with CF. One project goal was to identify unique miRs dysregulated by the various treatments of CFTR KD and/or As and investigate their role in innate immunity *in vivo* using the zebrafish model.

Deep sequencing analysis uncovered hundreds of aberrantly expressed miRs upon stimulation of the innate immune response. miRs were filtered based on high expression values
and increased or decreased log₂FoldChange. miR-199 was identified as our miR of interest for further investigation in the context of innate immunity.

Our initial investigation confirmed that miR-199 is upregulated in response to *P. aeruginosa* infection at 6 hpi (Fig. 4). Additionally, it was discovered via ISH that miR-199 expression is localized to the ear, heart and anterior kidney of the zebrafish (Fig. 5). The kidney is the site of hematopoietic stem cell origination in the zebrafish and is similar to the bone marrow of humans. Taken together, these data support the likelihood that miR-199 is indeed involved in the innate immune response. However, the specific pathway targeted by miR-199 remains to be fully understood.

In order to explore the role of miR-199, a functional approach was taken by implementing knockdown of miR-199 via morpholino injection at the 1-cell embryonic stage. Loss of miR-199 significantly decreased the susceptibility to *P. aeruginosa* infection and resulted in increased host survival after systemic infection (Fig. 6). Therefore, we hypothesized that loss of miR-199 may be regulating the function of phagocytes and their role in clearing infectious agents. Phagocytes play a vital role in clearing bacterial infection through phagocytosis and production of ROS. Zebrafish TargetScan identified the SFK Lyn as a potential target of miR-199. A recent study showed that *Lyn* knockdown using a *Lyn* morpholino had an inhibitory effect on the migration of neutrophils to the site of a wound in zebrafish. The study found that there was a roughly 30% decrease in neutrophils at the site of a wound upon knockdown of *Lyn* (Yoo et al). While the role of *Lyn* in response to wounds in the zebrafish has been investigated, studying the interaction between *Lyn* and miR-199 in response to bacterial infection provides a novel path for study. Although the relationship between miR-199 and *Lyn* is not fully understood, we hypothesize that miR-199 is targeting *Lyn* at the transcriptional level.
and interfering with the signaling cascade responsible for activation of phagocytes, specifically neutrophils.

Based on our observations, it is clear that miR-199 plays a role in the “fine-tuning” of the innate immune response in the zebrafish. The expression of miR-199 is clearly dysregulated upon stimulation of the innate immune system (Fig. 3). Although both decreased bacterial burden and increased survival are observed following miR-199 knockdown and *P. aeruginosa* infection, the biological mechanism underlying this result remains to be adequately described. Our preliminary results suggest that targeting of the SFK Lyn at the transcriptional level by miR-199 may be causing a dampened innate immune response. Future research is necessary in order to confirm this hypothesis. One avenue of investigation that is of particular interest is to perform qPCR measuring the expression of Lyn upon miR-199 knockdown during the course of *P. aeruginosa* infection. Another important observation would be to utilize an MPX zebrafish transgenic line having GFP-labeled neutrophils. These MPX fish could be injected at the 1-cell stage of development with miR-199 MO and then locally infected with *P. aeruginosa*. The migration of neutrophils to the site of infection could then be observed real-time and it could be determined whether miR-199 knockdown was playing a role in neutrophil migration. This specific investigation has a great deal of potential to provide insight into the innate immune response.

In conclusion, the investigation of aberrant miR expressions can help us gain a better understanding of gene regulation during the host innate immune response. If the specific role of miR-199 were completely understood, it could potentially serve as a novel therapeutic target for disease control in patients suffering from CF and chronic lung infection in which miR-199 expression could be modulated in order to combat its immune dampening effects.
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Author’s Biography:

Eliot Gagne was born on May 5, 1994 in Portland, Maine. Eliot grew up in Gorham, Maine where he graduated from Gorham High School in 2012. At the University of Maine, Eliot was a biochemistry major and an active member of the Honors College. He severed as the Vice President of both the Sophomore Owls Society and the Honors College Student Advisory Board. Eliot was inducted into Phi Kappa Phi Honors Society his sophomore year and into Phi Beta Kappa Honors Society his senior year. He spent a great deal of time at UMaine volunteering with the University Volunteer Ambulance Corps as well as at Eastern Maine Medical Center. He will continue his passion for medicine in the coming year at Tufts University School of Medicine in Boston, Massachusetts.

Outside of class, Eliot enjoys fly fishing in areas throughout Maine and especially at his camp on Nesowadnehunk Lake. He is also a fitness and nutrition enthusiast and spends a great deal of time researching and participating in fitness related activities. After medical school Eliot will serve as a lieutenant in the United States Air Force and plans to one-day return to his home state as a qualified and committed physician. Above all, Eliot is a proud brother, son, uncle and friend to many incredible individuals.