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Characterization of Genes in the CFTR-Mediated Innate Immune Response

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CHARACTERIZATION OF GENES IN THE CFTR-MEDIATED

INNATE IMMUNE RESPONSE

by

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Abstract:

Recently, the Kim Lab has shown that the cystic fibrosis transmembrane conductance regulator (*cftr)* gene is responsible for mediating resistance to *Pseudomonas aeruginosa* in a zebrafish infection model. Using the Gene Expression Omnibus, an NCBI functional genomics data repository, it was determined that Smad3, a transcription factor in the TGF-β signaling pathway, is upregulated in the presence of *P. aeruginosa*. It was found that in our zebrafish model, the Smad3 paralogs Smad3a and Smad3b are upregulated following microinjection of a *cftr* antisense morpholino oligomer. It was also found that microinjection of Smad3a and Smad3b morpholinos, along with a Smad2 morpholino, and subsequent infection with P*seudomonas aeruginosa* resulted in an increase in death, indicating that Smad3 has a protective effect against infection.

Acknowledgements

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THESIS INTRODUCTION:

The goal of this thesis is to further investigate and characterize cystic fibrosis (CF), an autosomal recessive multi-organ disease that is the result of a mutation in the *cftr* gene. The *cftr* mutation results in a nonfunctional form of a chloride channel that is essential for maintaining an ion balance within multiple organs. As a result of this *cftr*-related imbalance, many functional problems can occur in these organs. A characteristic condition in patients with cystic fibrosis is thick mucus in the respiratory tract that becomes prime breeding ground for bacteria (12, 18, 27).

The *cftr* mutation also affects the innate immune system, the part of the immune system that is the first to respond to infection. Because of this deleterious effect on the innate immune system, patients with cystic fibrosis are especially susceptible to bacterial infection. A pathogen that exhibits unusually high levels of infectivity in cystic fibrosis is *Pseudomonas aeruginosa*, a bacterium that is able to colonize the mucus of cystic fibrosis patients due to its anti-phagocytic activity and resistance to antimicrobials (12, 22). Further characterization of *cftr* is necessary in order to determine why it is only *Pseudomonas aeruginosa* that exhibits this high level of bacterial burden.

We are interested in characterizing some of the other genes that are also affected by this *cftr* mutation. The Kim Lab is involved in a collaborative effort to retrieve a list of candidate genes that are affected by the *cftr* mutation, *Pseudomonas aeruginosa* infection, and arsenic exposure. Since these data are not yet available, previously published data was used to investigate the link between the *cftr* mutation and *Pseudomonas aeruginosa* infection. While amassing my own list of candidate genes, I

identified Smad3, a transcription factor that is instrumental in regulating genes in the TGF-β signaling pathway, as a possible candidate gene. I wanted to investigate how the cystic fibrosis condition or bacterial infection could play a role in the expression levels of Smad3. Conversely, I wanted to investigate the effects of knocking down Smad3 in conjunction with a *Pseudomonas aeruginosa* infection.

LITERATURE REVIEW:

Danio rerio as a model organism

The zebrafish, *Danio rerio,* is a freshwater fish that has become increasingly exploited as a model organism. The development of zebrafish as a model organism began in the 1970s, when George Streisinger was looking for a vertebrate model to study the development of the nervous system, as well as to examine mutation-based genetic analysis. Zebrafish have a low cost of maintenance, ease of maintenance, and have large clutch sizes (11).

The genome of *Danio rerio* has been sequenced and has a high degree of gene similarity to *Homo sapiens* (6). Due to this similarity, human diseases like cystic fibrosis, muscular dystrophy, and several types of organ diseases, can be studied in the zebrafish model. Zebrafish has particular usefulness as a model organism in the studies of the innate immune system because it takes the zebrafish about 4 weeks to develop an adaptive immune system. Therefore, it is possible to look exclusively at the innate immune system (6, 32). Another advantage of the zebrafish as a model organism is its ability to be genetically modified in order to display certain characteristics, such as GFP-

tagged neutrophils. Antisense RNA technologies like morpholino oligomers are also used in the zebrafish model, in which a loss of function effect can be produced either by knocking down translation or blocking the splicing of the mRNA (34).

Zebrafish are useful for infection models like *Pseudomonas aeruginosa,* where both localized and systemic infections can be observed. One of the most defining features of using the zebrafish as a model for bacterial infection is its optical clarity, and the ability to look at infection real-time. Its optical clarity is important for certain injections, including into the Duct of Cuvier and into the hindbrain. Real-time analysis pertains to the idea that during the first week post fertilization, infections can be visualized *in situ* using fluorescent proteins on the bacterium (35).

Cystic Fibrosis and the *cftr* gene

Cystic fibrosis is among the most common genetically inherited diseases in the world, primarily affecting those of European descent. It is the most common lethal autosomal recessive disease in the white population, and affects about 1 in 3500 Americans (27). Cystic fibrosis is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*cftr)* gene, which codes for the CFTR protein, a chloride channel that resides in the cell membrane of epithelial cells. The most common change to this gene is F508del, a deletion of the nucleotides CTT in the gene, causing the loss of phenylalanine from the protein (27). There are a number of other mutations that can lead to cystic fibrosis, and some appear to be more prevalent in different populations. Due to the deletion of phenylalanine, the protein will misfold and be targeted for

degradation by the ubiquitin-proteasome pathway. Therefore, it will never reach the cell membrane (36). Other mutations of *cftr* can result in the protein reaching the cell membrane, but it will not be entirely functional (1, 3).

The CFTR protein is a chloride channel that is present in epithelial cells and is responsible for the conductance of chloride in and out of these cells (Figure 1). CFTR is a member of the ATP binding cassette protein family, in which the defining characteristics of this family are two membrane spanning helices and two nucleotide binding domains. The phenylalanine deletion in *cftr* occurs in the region of the gene corresponding to nucleotide binding domain one (NBD1). CFTR also contains a regulatory domain, making it unique among the ABC proteins (15). When unphosphorylated, the regulatory domain has a disordered shape, and prevents dimerization of NBD1 and NBD2, an action that is necessary to open the channel. When phosphorylated, the R domain assumes a more orderly structure, and allows for dimerization of NBD1 and NBD2 (2). Phosphorylation of these dimerized domains allows for the passing of chloride through the channel.
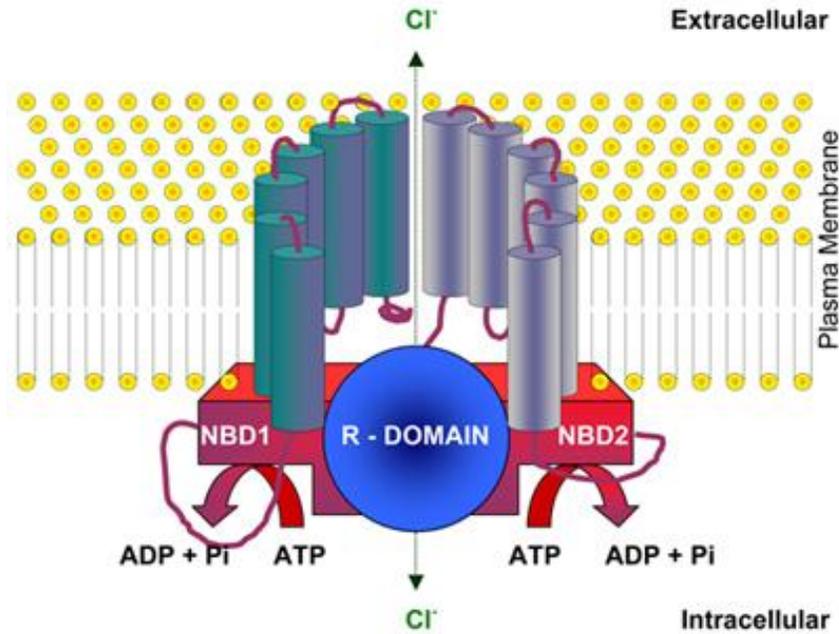
Figure 1. The CFTR channel as it is shown in an epithelial cell membrane. Phosphorylation of the R domain, NBD1, and NBD2 are all required in order to open the channel for chloride passage.

The CFTR protein is present in epithelial cells such as lung, liver, pancreas, and skin cells. The F508del mutation has a devastating effect on airway epithelial cells, where if it is not present to transport chloride across the cell membranes, a sodium-chloride ion imbalance results (33). This imbalance also affects the water content in these airways, affecting airway surface liquid, mucus transport, and will also result in thick mucus as a result of mucus dehydration. Thick mucus clogging the airways is one of the most common characteristics of cystic fibrosis, and it is very difficult for the cilia of these epithelial cells to clear this mucus from the airways. This mucus becomes an optimum media for pathogens like *Pesudomonas aeruginosa* to inhabit and colonize. Constant

infection and inflammation can lead to complications like pneumothorax, hypertension, and eventually death (18).

Currently, there is no cure for cystic fibrosis, but treatments and therapies are available to help lessen the complications that come with the disease. There are drugs available, such as Kalydeco ™ that can help people with the G551D mutation in the *cftr* gene. In this condition, the protein is transported to the membrane, but does not function. This drug helps to activate the NBDs and R domain to allow chloride transport. There are ongoing clinical trials with other drugs to investigate a treatment for F508del. Other treatments and therapies include antibiotics, chest physiotherapy, and mucus thinners (9).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic pathogen that is particularly invasive and problematic in patients with cystic fibrosis. It is a Gram-negative bacterium, with a membrane that has very low permeability, allowing it to have exceptional resistance to many antimicrobial drugs. *Pseudomonas aeruginosa* has the ability to secrete virulence factors via the Type III secretion system, where it relies on contact with epithelial cells. Such virulence factors lead to altered immune responses and cell death (8).

Pseudomonas aeruginosa has a limited number of porins, as well as a number of secondary mechanisms to prevent death of the cell. Secondary mechanisms, such as β-lactamase production and overexpression of efflux pumps, combine with the low permeability of the membrane to ensure that antibiotics are unable to act upon the organism. Also, mutations can lead to either increased resistance or increased

susceptibility to these antibiotics, although typically such mutations would lead to something like the overexpression of efflux pumps and reduced uptake (4). In addition to mutations, horizontal gene transfer via transposons and plasmids will also confer additional resistance to *Pseudomonas aeruginosa* populations. In these gene transfers, targets of antibiotics will usually be modified slightly in order for them to be unrecognizable by the antibiotics (4, 8).

In patients with cystic fibrosis, the link between antibiotic resistance and virulence often leads to respiratory failure and lethal complications. A hallmark characteristic of *Pseudomonas aeruginosa* is the formation of biofilms, in which a congregation of cells grows to create a community of antibiotic resistance, as well as increased resistance to the natural immune system mechanisms from the host. One of the most important aspects for chronic *Pseudomonas aeruginosa* infection is its ability to switch classes, from the biofilm, or mucoid, form of growth to the nonmucoid form of growth (Table 1). Each class has its own specific advantages, ranging from anti-phagocytic activity to antimicrobial resistance. (8, 12)

Property	Mucoid phenotype	Nonmucoid phenotype
Location in the lungs	Respiratory zone and conductive zone in sputum	Conductive zone in sputum
Biofilm formation *in vitro*	Yes	Yes
Biofilm formation *in vivo*	Yes	No
Multiply antibiotic resistance due to conventional mechanisms	Seldom	Frequent
Resistance (tolerance) due to biofilm properties	Yes	No
Responsible for lung tissue damage	Yes	No
Induces pronounced antibody response	Yes	No

Table 1. The above table shows the advantages between each phenotype, and how it is particularly advantageous to switch between these phenotypes.

In the cystic fibrosis condition, airway mucus becomes thicker and is harder to clear from the airways by cilia. This mucus becomes an ideal colonization medium for

Pseudomonas aeruginosa. The formation of biofilms in patients with cystic fibrosis in this mucus exacerbates inflammation and airway blockage, causing respiratory damage Chronic inhabitation of these airways by the bacteria results in an overzealous neutrophil response, leading to an increase in respiratory tract damage. Constant infection results in the activation of pro-inflammatory cytokines, such as IL-8, and other pro-inflammatory elements, such as ICAM-1. Neutrophil elastase is directly responsible for damaging the airway wall by degrading elastin. Reactive oxygen species and defensins are meant to help clear the infection, but end up being contributors to the scarring of the lung tissue. Respiratory failure eventually results due to constant infection (7, 33).

The Innate Immune System

The immune system is a collection of different types and systems that are in cooperation to ward off infections by bacteria and viruses. It can be broken up into the innate and adaptive immune systems, where innate immunity is primarily composed of non-memory related cells, while adaptive immunity has the capability to recognize a returning pathogen or infection, and a quicker, more specific defense can be launched (Table 2) (21).

	Innate Immunity	Adaptive Immunity
Components and Cells Involved	Physical and Chemical Barriers Phagocytic leukocytes Dendritic Cells NK Cells	Cell Mediated Immunity via T Cells Humoral Immunity via B Cells
Response Time	Immediate	Much slower (days)
Specificity	General	Very specific to certain antigens
Memory	No	Yes – memory B Cells have the ability to "remember" previous antigens

Table 2. The comparisons between innate and adaptive immunity (21, 23).

Although the innate immune system is not more specific than the adaptive system, it does provide the first line of defense for the body. This line of defense comes primarily in the form of mast cells, macrophages, neutrophils, natural killer cells, and dendritic cells (23). Each of these types of cells can perform different functions, whether it is phagocytosis, the release of certain inflammatory cytokines, or the recruitment of other members of the immune system. Together, the cells of the innate immune system are able to form a comprehensive first line of defense, while the more specific adaptive immune system is given time to react.

Some of the first responders to a site of infection are the neutrophils, which act to defend the body by phagocytosis and the release of various cytokines and granules (7, 33). The way in which neutrophils and other macrophages differ is through the presence of granules in neutrophils, which contain various enzymes like lysozyme, myeloperoxidase, and NADPH oxidase (21, 23). Neutrophils and macrophages are able to recognize their targets to be phagocytized is by the utilization of pattern recognition

receptors (PRRs). PRRs recognize and bind to pathogen-associated molecular patterns, PAMPs. Common PRRs include the Toll-like receptors (TLRs), mannose receptors, CD14, which is primarily found on macrophages, and NOD-like receptors, among others (21).

The TLR family of receptors contains multiple members, all of which are capable of recognizing different bacterial of virus components (Figure 2). TLRs are transmembrane receptors that contain a leucine rich repeat domain responsible for recognition of PAMPs, as well as an intracellular domain that is responsible for signal transduction. Some of the molecules that are recognized by TLRs include flagellin, lipopolysaccharide, ssDNA, and peptidoglvcan (21). Toll-like receptor 4 is responsible for recognizing lipid A, a component of the cell wall of *Pseudomonas aeruginosa.* Recognition of this lipid results in a pro-inflammatory response, which includes the release of various cytokines and chemokines that can increase blood flow, induce proliferation of immune cells, and recruit neutrophils to the site of infection (5, 10). Toll-like receptor 5 is another TLR responsible for the recognition of *Pseudomonas aeruginosa*. TLR5 is able to identify flagellin, the subunit that makes up the flagellum of *Psuedomonas aeruginosa* (22, 30).
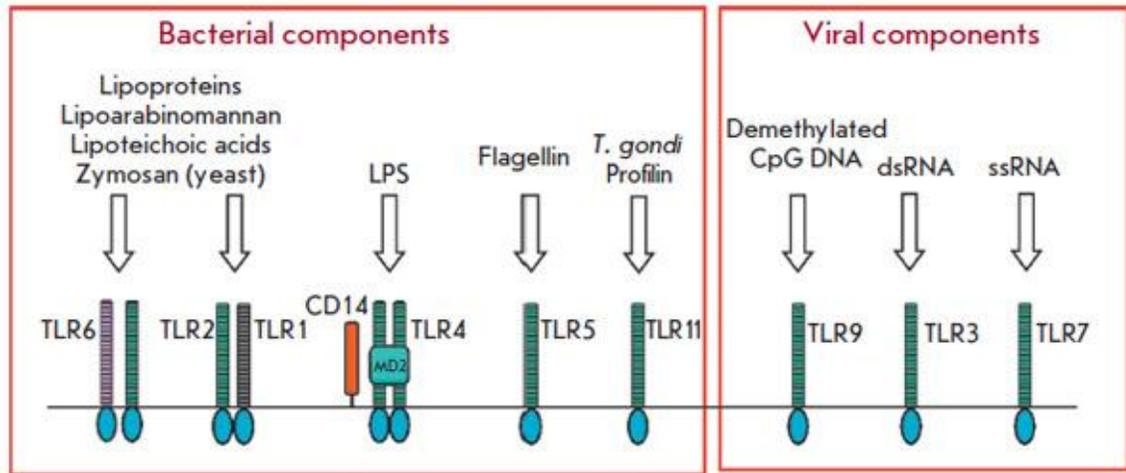
Figure 2. Shown are mammalian TLRs with known ligands, represented as they would be displayed on the cell surface (30).

In response to a component like LPS or other bacterial-specific molecules, a series of inflammatory events occur in order to reduce the potential hazardous effects of the pathogen. In particular, IL-1, IL-6, IL-18, IL-12, and TNFα can all work together to increase the inflammatory response. Such a response would increase body temperature thereby reducing the capability of the pathogen to replicate, as well as increase the migration of neutrophils and other macrophages to the site of infection via chemotaxis (21).

TGF-Beta Signaling Pathway

The transforming growth factor beta, or TGF-β, signaling pathway is an integral part of cell signaling within the cell. The TGF-β pathway is host to a number of different functions through ligand-activation of receptors on the cell membrane, phosphorylation

of transcription factors called Smads, and shuttling of these Smad transcription factors to the nucleus to regulate gene transcription (Figure 3). Among the notable functions of the TGF-β signaling pathway are regulation of the cell cycle and apoptosis, as well as immunosuppression and inflammatory and anti-inflammatory cytokine regulation (13, 14, 25, 26).
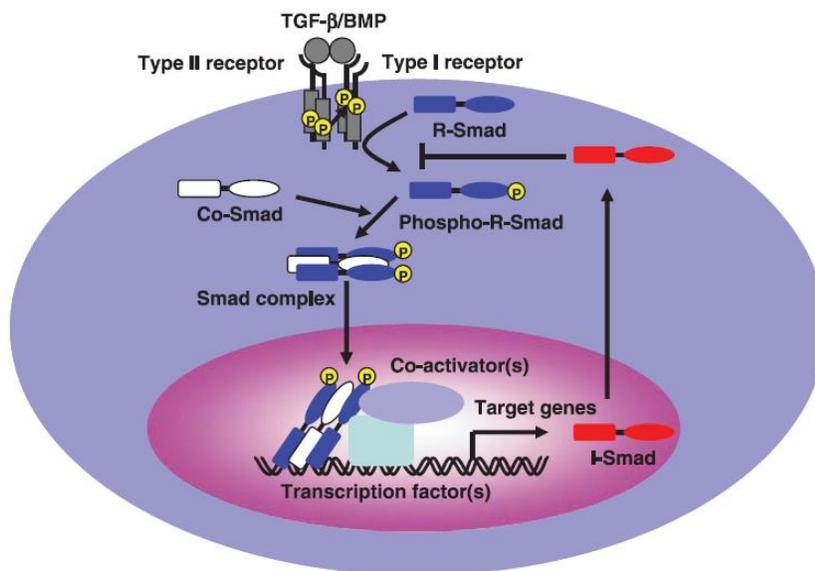
Figure 3. An overview of the TGF-β pathway. The binding of ligands to their appropriate receptor results in phosphorylation of Smad transcription factors. These transcription factors are shuttled to the nucleus, where they bind target DNA and result in the transcription of that gene (13).

The pathway begins with the binding of a ligand to the TGF-β receptors found on the cell membrane. These ligands consist of two different families: the TGF-β/Activin/Nodal family of ligands and the BMP/GDS/MIS family of ligands. Although each family, and each ligand itself, elicits a different response, they are characterized by

6 cysteine residues within the ligand. The role of these cysteine residues is to form intramolecular and intermolecular disulfide bridges. All of the TGF-β ligands exist in a pro-peptide form that is cleaved into the mature form. The function of the pro-peptide can vary between the different ligands, but primarily, it exists to serves as a scaffolding protein. It can also play a role in ligand stability (25). These ligands are shuttled to the cell membrane of target cells, where TGF-β receptors await to receive and transduce the message.

There are two different families of TGF-β receptors, the type I receptors and type II receptors. The BMP family of ligands shows high specificity for type I recptors, and low specificity for type II, while the TGF-β family typically only interacts with type II (31). The receptors are made up of an extracellular N-terminus domain, which binds the ligand, a transmembrane domain, and an intracellular C-terminus domain. Unique ligand binding domains on the surface of type I and type II receptors ensure that the proper ligands are bound to the right receptor. BMP homodimers interact with their type I receptor via hydrophobic interactions. TGF-β ligands also form homodimers, forming large receptor complexes with type II receptors, followed by complexing with type I receptors (31).

Upon binding of TGF-β ligands to the type II receptor, an interaction between type I and type II receptors occurs (Figure 4). A key structural difference between the two types of receptors is that the type I receptor contains a glycine-serine (GS) domain at its C-terminus, which is important for the type I/type II receptor interaction. Type II receptors contain a serine/threonine kinase domain at their C-terminus, which targets

serine and threonine residues in the C-terminus of type I receptors, specifically the

TTSGSGSG sequences in this domain (26, 31).
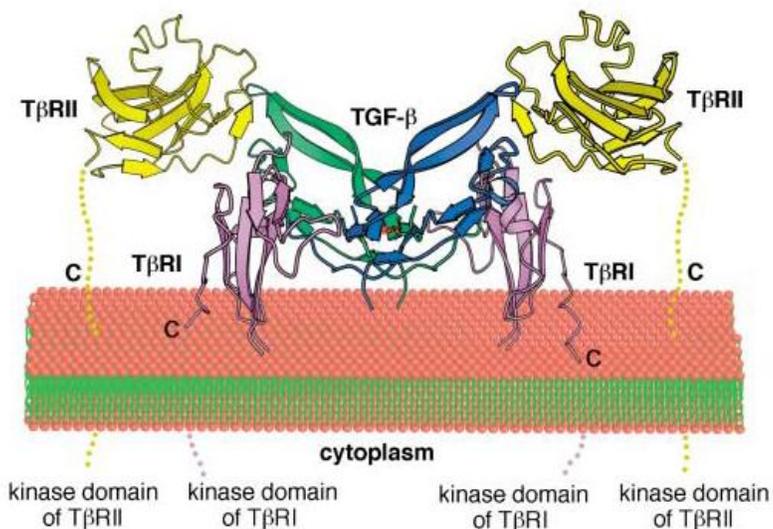
Figure 4. The TGF-β ligand (blue and cyan) forms a homodimer that initially

binds to the type II receptor ligand binding ectodomain (yellow). Then, it is able to also

bind to the type I receptor (purple), forming the large receptor complex. The binding and

proximity of the type I and type II receptors allows for phosphorylation and signal

transduction (31).

A phosphorylated type I receptor also acts as a kinase, phosphorylating

transcription factors called Smads. In humans, there are 8 Smad proteins, belonging to 3

different families, consisting of receptor-activated Smads 1, 2, 3, 5 and 8, co-Smad4, and

inhibitory Smad7 (26). Structurally, Smad proteins have 2 conserved domains. The MH1

domain is the N-terminal domain, and is primarily responsible for sequence-specific

DNA recognition. The MH2 domain, at the C-terminus, directly interacts with nuclear

pores, allowing access into the nucleus. It also regulates oligomerisation with other

Smads (26). An SxS motif in the MH2 domain is critical for receptor recognition.

Phosphorylation of this domain by the type I receptor is required for Smads to be translocated to the nucleus. Disruption of this motif would result in altered levels of Smad signaling (26).

R-Smads are recognized by the type I receptor kinase by two structural characteristics. A basic patch of amino acids on the surface of R-Smads allows for binding of the phosphorylated GS domain on the type I receptor to Smad (26). The specificity of binding results from the interaction between the L45 loop of the type I receptor and the L3 loop of R-Smads (31). As a result, TGF-β receptors only recognize Smad2 and Smad3, while BMP receptors recognize Smad1, Smad5, and Smad8. The specificity within the receptors comes down to matching sets of receptor loop L45 and Smad loop L3. Smads can be localized at the cell membrane next to TGF-β receptors by the Smad anchor for receptor activation, SARA. SARA binds to Smad2 or Smad3 via a hydrophobic surface region (31).

Once localized to the membrane, Smad is ready to be phosphorylated by TGF-βR1. Phosphorylation of the SxS motif in the MH2 domain of R-Smads results in dissociation from SARA. This phosphorylated motif is then able to interact with other Smad proteins, either forming homomers or heteromers. Smad3 will also associate with Smad4, the co-Smad, in order to assist with translocatation to the nucleus (37). All of the Smads contain their own nuclear localization sequences, which are able to bind to specific importin proteins that transport them into the nucleus (26).

With the R-Smad in the nucleus, it is nearly ready to promote transcription of its target gene. Smads bind to DNA with sequence specificity. The Smad binding element,

located on the target DNA sequence, at minimum, requires the sequence 5'AGAC 3', but will typically be recognized by Smad with additional bases on either end (Figure 5). R-Smads achieve specific target DNA binding due to cell-type specific cofactors, including co-activators and co-repressors, as well as other Smads and DNA binding elements. The association of Smad to an array of molecules results in the transcription of specific genes (25).
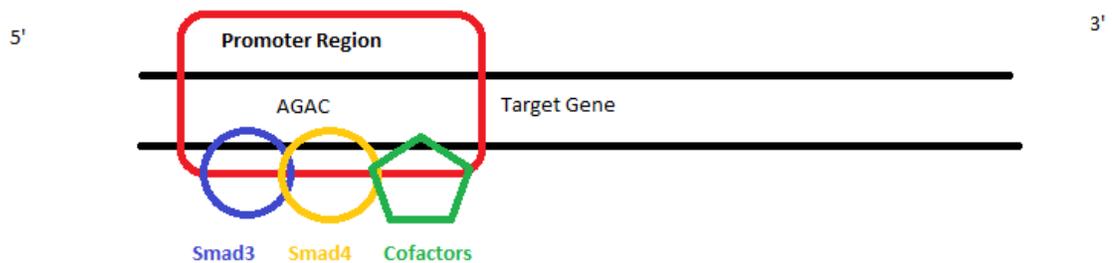
Figure 5. A simplified diagram of Smad3 associating with Smad4 and other cofactors, binding to its target sequence in the promoter region. This gene would have transcription machinery recruited to it.

In short, Smad3, and all transcription factors, facilitate transcription of these target genes by binding to their target sequence in the promoter region of the target segment of DNA. Essentially, this will either promote or repress transcription of these genes by stabilizing or destabilizing the DNA, affecting the way in which RNA polymerase is able to bind to the DNA. In the case of Smad3, binding to its target gene will actively recruit transcription machinery to the target, resulting in the transcription and eventual translation of the gene.

Smad signaling can be either positively or negatively regulated through direct competition for binding sites, as well as through the ubiquitin-dependent degradation pathway. The direct competition for binding sites on TGFβ receptors, R-Smads or co-Smad4, is achieved via binding by c-Ski, Smad6, or Smad7 (19). C-Ski negatively regulates Smad signaling by competing for the MH2 domain on Smad4. When interfering, a complete Smad complex is unable to form, and as a result, the transduction from the receptor is unable to continue. I-Smad7 does not directly interfere with any of the R-Smads, but instead acts by binding to TGFβR1, and in the process, inhibits phosphorylation of the R-Smads. Smad6 is able to bind to R-Smads, preventing the formation of a complete complex, similar to c-Ski (31).

Another method of inhibition is through a variety of ubiquitin-dependent degradation pathways, including the E3 ubiquitin ligases Smurf and APC. These ligases have target proteins that associate with the Smads, which can include c-Ski and SnoN. SnoN is able to activate the ubiquitin-mediated pathway of degradation by associating with the Smad that is to be degraded. SnoN can either downregulate or upregulate the pathway, depending on which Smad it binds to. It recruits Smurf1 and Smurf2, which are Smad ubiquitin regulatory factors. These Smurfs induce degradation of R-Smads by the ubiquitin-proteasome pathway (13).

Targets of Smad3, the Immune System, and the Connection to Cystic Fibrosis

With the complexity and vastness of the TGF-β pathway being as large and encompassing as it is, it is no surprise that there are many targets of Smad3. Although targets of Smad3 and the TGF-β pathway usually play a role in cell cycle, apoptosis, or cytoskeleton rearrangement, there are still targets that can play either a direct or indirect role in the immune system. Among the most important targets of Smad3 in relation to the immune system are the anti-inflammatory cytokines IL-10 and RANTES, and the inflammatory cytokine IL-8 (14, 17, 19, 28). In normal lung epithelial tissue, the presence of TGFβ1 as a ligand and Smad3 would result in the upregulation of RANTES and downregulation of IL-8. The IL-8 promoter is Smad3-repressible; that is, when Smad3 is present, IL-8 will not be transcribed (17). But, previous studies have shown that the cystic fibrosis condition results in the knockdown of Smad3 in nasal epithelium. As a result, the anti-inflammatory cytokines for which it is responsible for regulating, IL-10 and RANTES, are downregulated, and since Smad3 is not present, IL-8 is able to be transcribed, resulting in an increased inflammatory condition (17).

Smad3 was found to target the gene responsible for encoding MARCKS, a protein that is responsible for actin rearrangement. This actin rearrangement helps to guide neutrophils to the site of infection (38). Since Smad3 appears to be knocked-down in cystic fibrosis-affected nasal epithelial tissue, MARCKS would also be affected, lowering the rate at which neutrophils are able to migrate to the infection site. This would potentially decrease the overall ability of the immune system to clear the airways of pathogens like *Pseudomonas aeruginosa*. The importance of Smad3 in relation to the cystic fibrosis condition remains to be seen. But, altered levels of Smad3 would most

certainly affect its immune targets, thereby affecting the organism's ability to handle and clear an infection.

INTRODUCTION

Cystic fibrosis is the most common autosomal recessive disorder in North America, affecting 1 in 3500 Americans (27). Under normal conditions, the cystic fibrosis transmembrane conductance regulator (CFTR) is responsible for chloride ion transport, and is found in the membranes of epithelial cells. In patients with cystic fibrosis, a mutation in the *cftr* gene exists. There are a multitude of mutations that can result in varying degrees of the disease, but the most common mutation is the F508 deletion, in which the phenylalanine residue at position 508 is deleted. The F508 deletion that occurs in nucleotide binding domain 1 of CFTR results in the misfolding of the protein, and due to this mutation, the protein will never reach the plasma membrane. As a result of the mutation, chloride ion transport across these epithelial cell membranes is severely affected. It also indirectly affects sodium ion transport, as well as water balance (7, 24, 27).

A characteristic condition resulting from the F508 deletion is thick and dry mucus due to the overall ion and water imbalance. This mucus becomes optimum breeding ground for many types of pathogens, particularly *Pseudomonas aeruginosa* (5, 29). *Pseudomonas aeruginosa* is problematic for patients with cystic fibrosis due to the bacterium's low membrane permeability, as well as its ability switch back and forth between multiple forms. Its low membrane permeability allows for increased resistance

to antibiotics, and its ability to switch between nonmucoid and mucoid forms allows for phagocytic resistance. In those with the *cftr* mutation, *Pseudomonas aeruginosa* is the pathogen that has been reported to have the highest bacterial burden (29). It is not entirely known why the *cftr* mutation causes increased susceptibility to *Pseudomonas aeruginosa* and not other bacteria.

In response to infection by *Pseudomonas aeruginosa,* the immune system responds with the migration of neutrophils. Neutrophils are granulocytic phagocytes that will release various degradative proteins in response to the infection. Such degradative proteins include elastase and lysozyme (22, 24). An overzealous immune response by these neutrophils results in scarring and inflammation in the lungs of cystic fibrosis patients. To help with the migration of neutrophils are various chemokines that can be released by cells that have come in contact with a foreign antigen (21). The regulation of some of these chemokines can be traced to the TGF-β signaling pathway. Although this pathway is more notorious for regulating process like cellular differentiation, apoptosis, and the cell cycle, some of its targets are immune related (13, 17).

A key part of the TGF-β pathway is Smad3, a transcription factor that is translocated to the nucleus and is responsible for binding to the promoter region of genes to be regulated. The immune-related targets of Smad3 include IL-10, an anti-inflammatory cytokine, and MARCKS, a protein that is responsible for actin cytoskeleton rearrangement to assist with neutrophil migration. Previous research has shown that the expression levels of Smad3 are altered in response to a knockdown of *cftr*¸ specifically in nasal epithelium (17). We are interested in whether Smad3 could play a protective role in defense against infection, and what its role in connection to *cftr* could be.

METHODS

Identification of Novel Gene Targets using NCBI's Gene Expression Omnibus

Gene targets for characterization were identified using multiple databases through NCBI's Gene Expression Omnibus tool. The databases selected for analysis and gene selection were: *Bronchial epithelial cell line response to various airway pathogens* (GDS2606), *Airway epithelial cell response to Pseudomonas aeruginosa rsmA mutant infection* (GDS2287), *Cystic fibrosis patients with mild and severe lung disease: nasal respiratory epithelium* (GDS2143), *Cystic fibrosis and small intestine* (GDS588) and *Cystic fibrosis pancreatic gene expression* (GDS567). Genes were selected based on their upregulation either in the presence of *Pseudomonas aeruginosa* infection or the presence of cystic fibrosis, depending on the database.

Zebrafish Maintenance

Zebrafish embryos were maintained in 50 mL of egg water (60 mg/L of Instant Ocean, Spectrum Brands, Madison, WI). Embryos were maintained at 28° C.

Antisense Morpholino Injections

Antisense morpholino oligonucleotide injections were performed on zebrafish embryos that were currently in the 1 cell stage (0.5-1 hpf). *Cftr* morpholino injections were performed with 3 nl, at 2.5 ng per embryo. Two different morpholinos were used: a splice blocker morpholino and translation blocker morpholino, as designed by *Phennicie*

et al (Table 3) (29) . *Smad3a* and *Smad3b* morpholinos were co-injected at 2 ng per embryo, along with a p53 morpholino at 12.5 ng, for a total injection volume of 3 nl. When performing *Smad2*, *Smad3a*, and *Smad3b* morpholino injections, 2 ng per embryo was once again used, with 18.75 ng of p53 per embryo. *Smad2*, *Smad3a*, and *Smad3b* morpholinos were designed by *Jia et al* (16).

Gene Name	Morpholino Sequence
Cftr (Translation blocker)	5′-CCACCTGTAAATATTCAGAGCAGAT-3′
Cftr (Splice blocker)	5′-CATCCTCCACAGGTGATCTCTGCAT-3′
Smad3a	5'-TTCAGTTCAGCGTTCCTCTATTGC-3'
Smad3b	5'-GCAATATAGACATCTTTAGTTGAT-3'

Table 3. Morpholino antisense oligonucleotide design for 1 cell injections, as synthesized by Gene Tools (Philomath, OR).

Bacterial Injections

Wild-type *Pseudomonas aeruginosa* PA14 was grown overnight in LB broth at 37° C. Liquid cultures were washed with phosphate-buffered saline, and cell density was determined using a spectrophotometer. *Pseudomonas aeruginosa* and PBS injections were performed 2 days post morpholino injection. To ensure a systemic infection, injections took place in the Duct of Cuvier. An injection volume of 3 nl was used for 250 CFU and 1 nl for 150 and 90 CFU.

RNA Extraction and cDNA Synthesis

RNA extraction was performed using the TRIzol phenol/chloroform extraction method (Invitrogen). RNA extractions were done in triplicate, with 10 fish in each replicate. Fish were homogenized into the TRIzol reagent, which was followed by an incubation period of 3 min. with chloroform and centrifugation at $4°$ C for 15 min. at 12000 g. RNA was isolated by adding 250 x linear acrylamide and 100% isopropanol to the aqueous phase, which was centrifuged at 15000 x g for 15 min. at $4°$ C. The RNA pellet was washed with 70% ethanol, spun at 15000 g for 5 min. and $4°$ C. The RNA pellet was resuspended into RNAse-free 10mM Tris-Cl, pH 8.0, and incubated at $50°$ C for 15 min. Extracted RNA was converted to cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA).

Quantitative PCR

Target gene primers were designed using Primer3 for quantitative PCR (Table 4). Fold induction values were determined using a CFX96 real-time detection system with cDNA synthesized from control morpholino and *cftr* morpholino injected embryos and SsoFast EvaGreen as the fluorescent dye for detection of expression (BioRad, Hercules, CA).

Gene name	Forward Primer (5'-3')	Reverse Primer (5'-3')
Smad3a	GCA ACT ACA TAC CAG AGA CGC C	TGG AGA ACC TGT GTC CAT GC
Smad3b	TGG ACA CAG GTT CTC CAA CG	TGG TTG TGA GGC ATG GAA AG
Vrk3	TGA ACC TTC ACC GCA ACA AG	GAG GAG TCC CCA GCA CAA TC
Ptpn2a	TGG AGG AGG CTC AAA GAA GG	TTG GAA CCC TGT TCC CAA AC
Ptpn2b	CTG GTT GAC ACT TGT CTT GTC TTG	TCT CGC CAT TGT TTC TGC AC

Table 4. List of primers designed for quantitative PCR.

Cloning of *Smad3a* and *Smad3b*

    Smad3a and *Smad3b* were cloned using the following primers: *Smad3a:*

CGTGAGAGACTGCGCAATAG (forward) and TACATTGGGCCAGATTTGTG

(reverse). *Smad3b* was cloned using heminested primers, which comprised of:

ATCATGCGGTGATGTGACTC (forward), GAGTCCAGAGGCTGTTTTGC (reverse,

outside) and TGAGTGAGCACCTTGTCCAG (reverse, inside). cDNA was used from

uninjected zebrafish. Desired genes were inserted into TOPO TA vectors (Invitrogen,

Carlsbad, CA) and transformed into NEB competent cells (New England Biolabs,

Ipswich, MA).

RESULTS

Upregulation of *Smad3a/Smad3b* in *cftr* Morpholino Injected Whole Fish Tissue

We wanted to investigate the effects that the *cftr* knockdown would have on the expression of our target genes. Quantitative PCR was performed using zebrafish that had been injected with the *cftr* morpholino. Analysis of gene expression in this whole fish tissue indicates that both *Smad3a* and *Smad3b* are upregulated in fish with lower expression levels of *cftr* (Figure 6). *Smad3a* shows a 1.36 fold induction, while *Smad3b* shows a 1.52 fold induction.
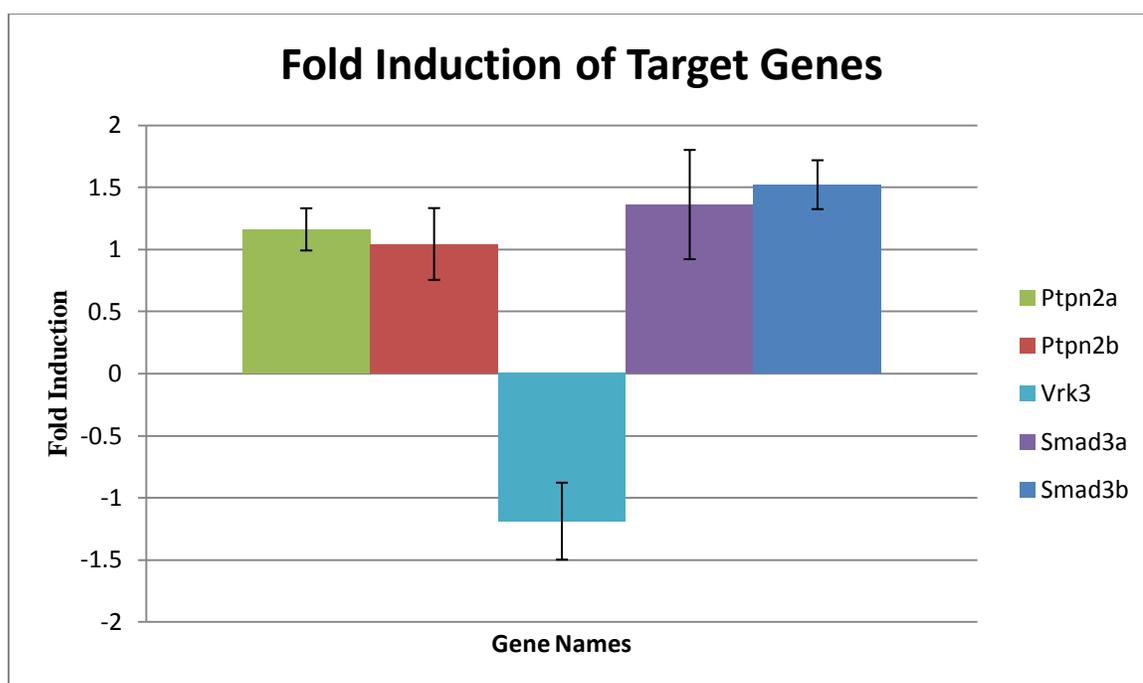
Figure 6. Quantitative PCR graphs of *cftr* morpholino injected fish. The activity of target genes in *cftr* morpholino injected fish was measured in comparison to injection with a control morpholino. All samples were normalized to beta-actin levels of expression.

Increased Death Associated with *cftr* Morpholino Injection in Conjunction with

***Pseudomonas aeruginosa* Infection**

     Phennicie et. al had previously performed bacterial burden studies with

Pseudomonas aeruginosa using *cftr* morpholino injected fish. They had shown that the

bacterial burden is higher in fish with the *cftr* knockdown (29). A mortality curve had not

been published, so we aimed to produce a curve showing that this increased bacterial

burden would correspond to an increase in death over control. An increase in death has

been shown, consistent with the previous research suggesting that *cftr* knockdown has a

negative-regulatory effect on the immune system (Figure 7).
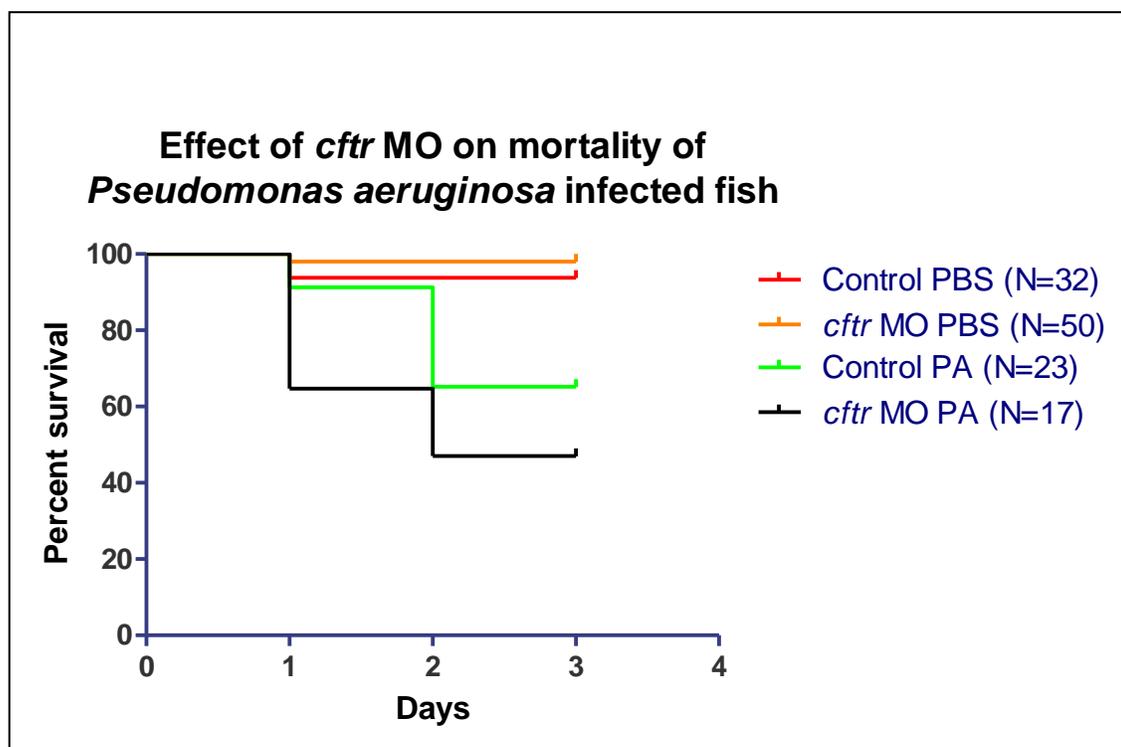
Figure 7. *cftr* knockdown and associated death in conjunction with *Pseudomonas*

aeruginosa infection. *Phennicie et al.* had previously proven an increase in bacterial

burden existed in fish with the *cftr* morpholino. Here it is shown that there is an increase

in death in morpholino injected fish with the infection at 150 CFU (unpublished data from *Sullivan*). Although there appears to be an increase in death, the P-value, as calculated by the Logrank test, is 0.1553, indicating there is this trend of death is not statistically significant.

Increased Death Associated with *Smad3a/Smad3b* Morpholino Injected Fish along with *Pseudomonas aeruginosa* Infection

Morpholinos of *Smad3a* and *Smad3b* were used to attempt to see if these genes had a role in survival of the fish upon infection with *Pseudomonas aeruginosa.* Embryos were also co-injected with a *p53* morpholino to ensure that off-target effects of the *Smad3a/3b* morpholinos were not induced. After 1 day of infection, there was a 13% increase in death among the *Smad3a*/*3b* morpholino injected fish in comparison to control fish (Figure 8). Further experiments need to be performed in order to more accurately determine if there is a significant increase in death of these fish due to the morpholino knockdown, and not just due to infection.

Effect of *Smad3a/3b* MO on mortality of *Pseudomonas aeruginosa* infected fish

Legend:
- Control + p53 PBS (N=20)
- *Smad3a/3b* MO + p53 PBS (N=25)
- Control + p53 PA (N=36)
- *Smad3a/3b* MO + p53 PA (N=21)
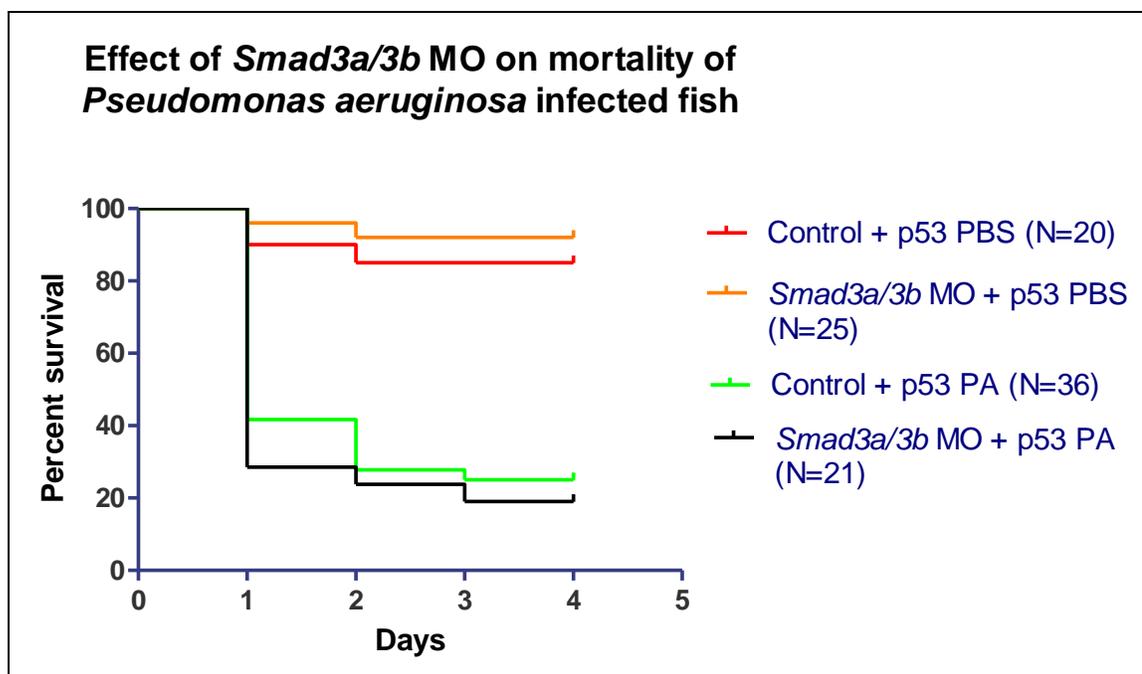
Figure 8.The percent survival of morpholino and *Pseudomonas aeruginosa* injected fish with 250 CFU. Shown is the apparent increase in death after 1 day of infection, indicating that in the very short term, *Smad3a* and *Smad3b* knockdown may contribute to the increased death of the fish. This trend of increased death was proved to be insignificant, as calculated by the Logrank test, resulting in a P-value of 0.5214.

We believe there to be a functional redundancy between Smad3 and Smad2, another transcription factor with similar roles in the TGF-β pathway. Another set of injections was performed, this time with the *Smad2* morpholino included (Figure 9). These injections resulted in a 20% increase in death in morpholino injected fish after day 1 and day 2 post infection.
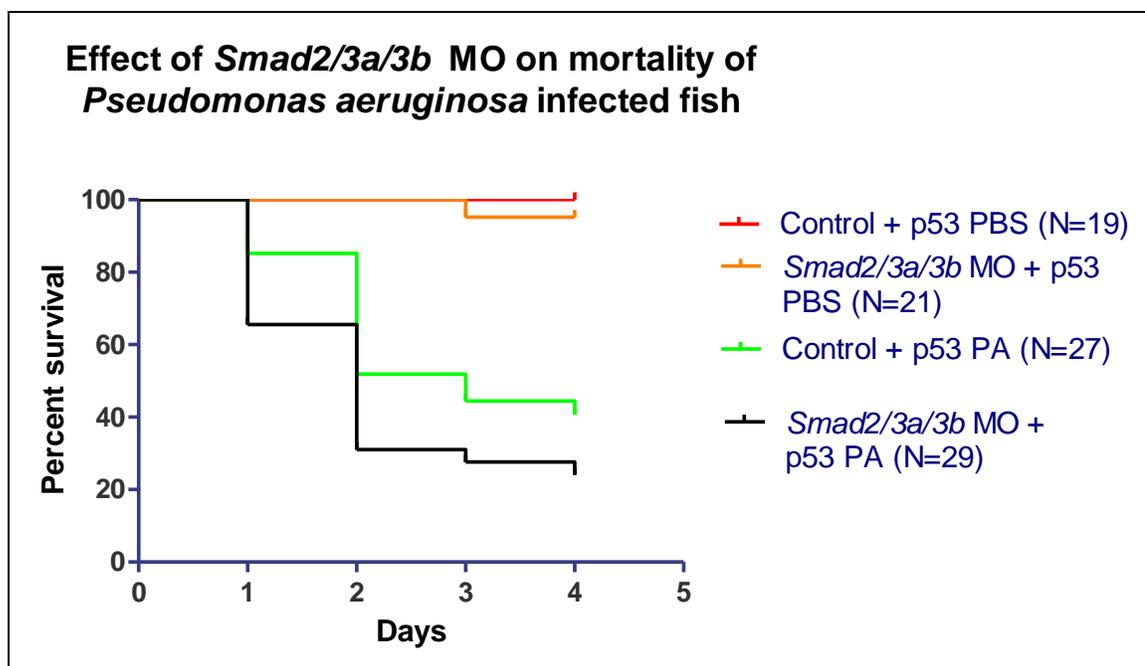
Figure 9. The percent survival of fish also injected with the Smad2 morpholino, at 90 CFU. In this graph, both the functional redundancy and amount of bacteria injected have been accounted for from the previous injection, resulting in a widened gap between control and morpholino injected fish. This trend was proved to be insignificant, as calculated by the Logrnak test, resulting in a P-value of 0.988.

Identification of Smad3 Target Genes

Smad3 target genes were identified using GEO, in which regulated genes specific to the immune system were identified (Table 5) (38). Although this is not an exhaustive list of target genes, analysis of these selected genes via qPCR will be performed to determine their levels of expression in *cftr* morpholino injected fish or *Pseudomonas aeruginosa* infected fish.

Gene	Function
TACR1	Substance P binding, control of innate immunity
WASPIP	Responsiveness to IL-2
Wdr34	TAK1-associated suppressor of the IL-1R/TLR3/TLR4-induced NF-kappaB activation pathway
PREP	Regulation of IL-10
YWHAZ	Role in phagocytosis and microbial resistance
TCF7	(Transcription factor 7 / T Cell Factor 1)Wnt pathway, regulation of expression of IL-17
MARCKS	Regulation of neutrophil migration
OPN3	Possible role in airway inflammation

Table 5. A selective list of Smad3 target genes, as identified in "SMAD3 targets in A549 lung epithelial cells, GSE26858" (38).

DISCUSSION

Use of Bioinformatics to Determine Gene Targets

This study began with the analysis of various databases that contained gene expression data from various experimental conditions. By cross-referencing these databases and conducting further literature review, a selective list of target genes was created to investigate further (Table 4). The use of bioinformatics, microarrays and deep sequencing data is quickly becoming the most efficient method to look at gene expression on a large scale. The Kim Lab is currently in the middle of a collaborative effort to analyze gene expression from zebrafish that have been exposed to arsenic in conjunction with *cftr* knockdown and *Pseudomonas aeruginosa* infection. Once these data are

released, another comprehensive set of genes will be available that can offer further insight into the cystic fibrosis condition.

Activity and Targets of Smad3

We have found that expression levels of both *Smad3* paralogs are upregulated in whole fish injected with the *cftr* morpholinos (Figure 6). It is also worth noting that *Smad2* was found to be upregulated in the same database that Smad3 was identified from, further strengthening our theory of a functional redundancy. Based on these data, and with the knowledge that Smad3 has potential to regulate inflammatory and anti-inflammatory responses, the increase in expression of *Smad3* could be due to the need for more anti-inflammatory cytokines. IL-10, an anti-inflammatory target of Smad3 could be expressed to prevent an overzealous inflammatory reaction by the rest of the immune system (17). MARCKS, an actin rearranging protein, may also be upregulated to help with the migration of neutrophils to the site of infection (38).

Previous research suggests that, in nasal epithelium with the expression of *cftr* knocked-down, Smad3 also is knocked down (17). It should follow that the previous anti-inflammatory response described above would not occur in nasal epithelium tissues with the *cftr* knockdown. Instead, an increase in IL-8, an inflammatory cytokine, was observed in these tissues. The knockdown of *cftr,* and therefore Smad3, could play a role in exacerbating the inflammatory response in patients with cystic fibrosis by increasing the levels of IL-8 produced and decreasing the levels of IL-10 produced.

By recognizing that Smad3 has differential expression due to the *cftr* knockdown, it will be worthwhile to work with the targets of Smad3 to attempt to determine if these targets are also differentially expressed (Table 5). With the knowledge that a *cftr* knockdown also results in an increase in death, it is possible that the differential expression of these targets could contribute to this death.

Using a Tetracycline Inducible Promoter to Examine Spatial and Temporal Expression

In order to examine organ specific expression of Smad3 in zebrafish, tetracycline-inducible promoter system will be utilized. Thanks to the Weidinger Lab, (Biotechnology Center and Center for Regenerative Therapies, Dresden, Germany) plasmids have been obtained containing promoters with the capability of being turned on and off in the presence or absence of tetracycline (20). A *cftr* promoter will be used in this plasmid to drive the expression of a dominant negative Smad3b gene. In the dominant negative Smad3b, the phosphorylation site that is necessary for translocation to the nucleus has been disrupted. Therefore, only tissue that contains the *cftr* gene will express dominant negative Smad3b. Desired experimental variables, such as the injection of the *cftr* morpholinos and *Pseudomonas aeruginosa* infections, will be applied in order to see the direct relationship between *cftr* and Smad3.

The *cftr* mutation resulting in cystic fibrosis is known to compromise the immune system of these patients, although it has never been clearly identified how. Increased neutrophil presence in the airways and the increase of inflammatory cytokines are

normally signs of fighting an infection, but the vast majority of patients with cystic fibrosis still possess compromised immune systems. *Pseudomonas aeruginosa* is a bacterium that is able to take advantage of this condition, ultimately leading to death of the host. By further characterizing *cftr* and the genes affected by the mutation or *Pseudomonas aeruginosa* infection, it is hoped that therapies can be discovered and introduced to develop a cure for cystic fibrosis.

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Author's Biography

Eric M. Peterman grew up in Mendon, Massachusetts, where he attended Nipmuc Regional High School. Upon graduating, he initially chose University of Maine for their biology program, but decided that majors in biochemistry and molecular biology would better suit his interests. Apart from being involved in the Honors College, Eric has been involved in the Club Volleyball team, serving as treasurer and vice president.

Following graduation, Eric is returning to the University of Maine for his Master's Degree in microbiology, which he will use to gain entry into either a PhD program or employment.