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EFFECTS OF ARSENIC RESPONSIVE *P21* ON INNATE IMMUNITY AND
APOPTOSIS IN ZEBRAFISH

by

Gabriel O. Vachon

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
Biology

The Honors College

University of Maine

May 2013

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Abstract

Arsenic is a heavy metal that is frequently found in drinking water, especially well-water in the Northeastern United States. Arsenic is thought to be involved with numerous negative health conditions. This project examined the impact of this environmental toxicant, on the zebrafish innate immune system. Specifically, this project characterized the gene encoding for the protein p21. The particular gene is *cdkn1a* (will be referred to here by its product "p21" both for the protein and gene itself which will be italicized) and had increased expression as a result of arsenic exposure from a previously complete microarray analysis. In attempt to determine possible results from *p21* induction, this project examined how it interacts with apoptotic and immune genes. This study increases our understanding of crucial apoptotic and cell cycle regulation pathways that may be altered as a result of exposure to 'safe' arsenic concentrations.

Acknowledgements

I would like to thank all members of my committee for their respective contributions to my final thesis. I appreciate all of the insight, assistance, and guidance provided by Dr. Carol Kim and all of those who work in her lab. These folks include, but are not limited to, Dr. Con Sullivan, Eric Peterman, Rickey Luc, Meghan Breitbach, Michelle Goody, Dawn Sullivan, Kristin Gabor, Juyoung Shim, Ray Luc, and all members of the DNA Sequencing Facility. I would also like to extend my appreciation for funding and support provided through the Ro1 (#5405558) and COBRE (#5405597) grants. This thesis would not have been possible without the support from the aforementioned members and contributions along with all others whom I may have mistakenly omitted. Thank you very much!! Enjoy.

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I. THESIS INTRODUCTION

The goal of this thesis was to investigate the role that arsenic plays in the regulation of the cell cycle. It was found that arsenic exposure at levels considered to be safe for drinking water in the state of Maine according to the US Environmental Protection Agency and the World Health Organization have a inductive effect on expression of the cell cycle regulating gene encoding for the protein *p21*. The purpose of my work was to gain an understanding of potential roles that this gene plays as it interacts with other genes of similar function based on previous studies. Various tests were performed to ensure that the function of *p21* in zebrafish is comparable to that in other vertebrate animals and to characterize the effects of *p21* on the little known *tnfaip8L1* "*TIPE1*" gene.

Analysis of RNAseq data collected by Kim Lab members indicated that arsenic exposure can lead to an increased in the expression of *p21*. *p21* is a known cell cycle regulator and is intimately involved in apoptotic pathways as well (3). How does induction of *p21* affect the physiology and immune response of zebrafish, particularly as this applies to apoptosis and the regulation of cell proliferation? In order to explore the role that *p21* may have in the innate immune response and apoptotic regulation, techniques were employed to promote the expression of *p21* or to inhibit the expression of *p21*. Expression levels of genes involved in *p21* pathways were then determined after induction or repression of *p21*. With these techniques, a number of pathways and feedback loops were investigated in depth.

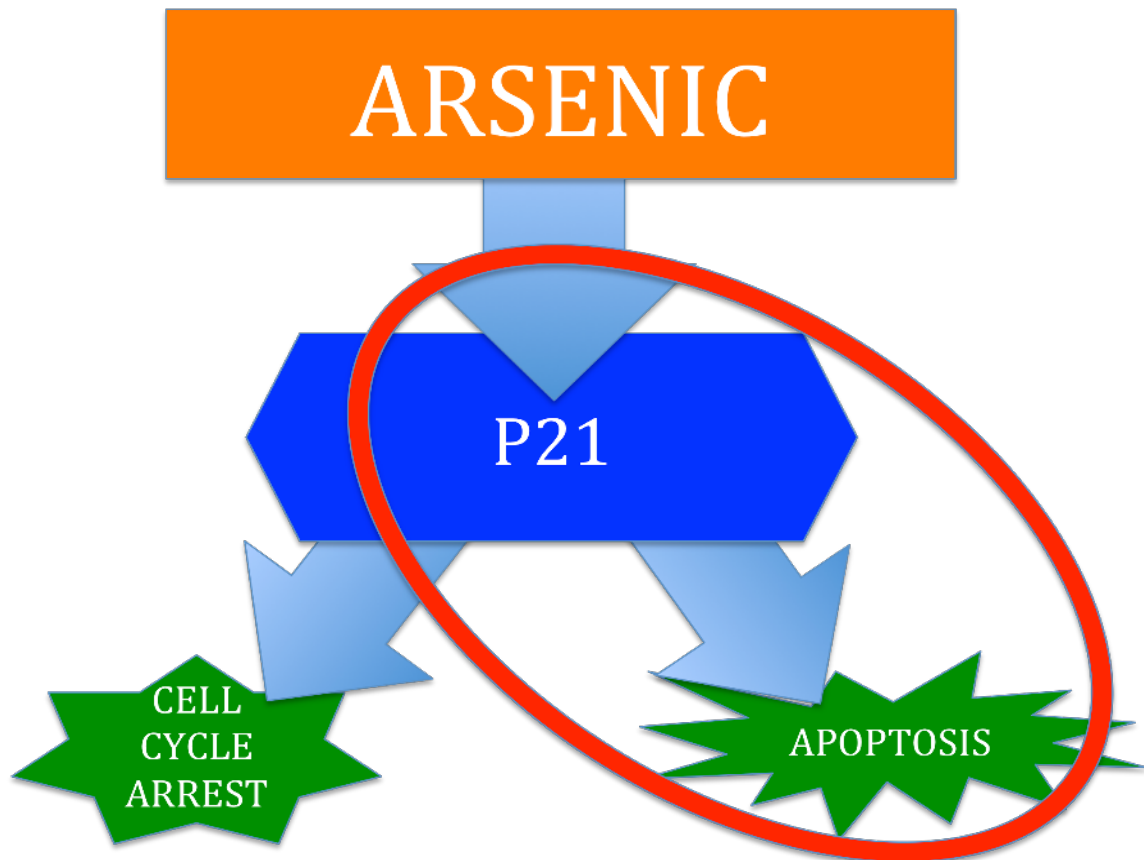


Figure 1: Arsenic induces *p21*. This project focuses on *p21* and apoptotic pathways.

The zebrafish was used as a model organism to study on the function of *p21*. Experiments are designed to study underlying genetic changes that alter apoptotic and cell cycle regulatory processes. A number of genes were studied, including *p21*, *cse1L*, *p53* (referring to the gene encoding for the protein product "p53"), *TIPE1*, *PUMA*, *CFTR*, and *C-MYC* with emphasis on *p21*, *p53*, *TIPE1*, and *PUMA* results. *p53*, *PUMA*, and *p21* are three genes that are involved with apoptotic pathways. These genes interact to regulate cell cycle arrest and apoptosis. *TIPE1* is a previously uncharacterized gene belonging to the *tnfaip8* family, and is presumed to play a role in apoptosis (see figure 2).

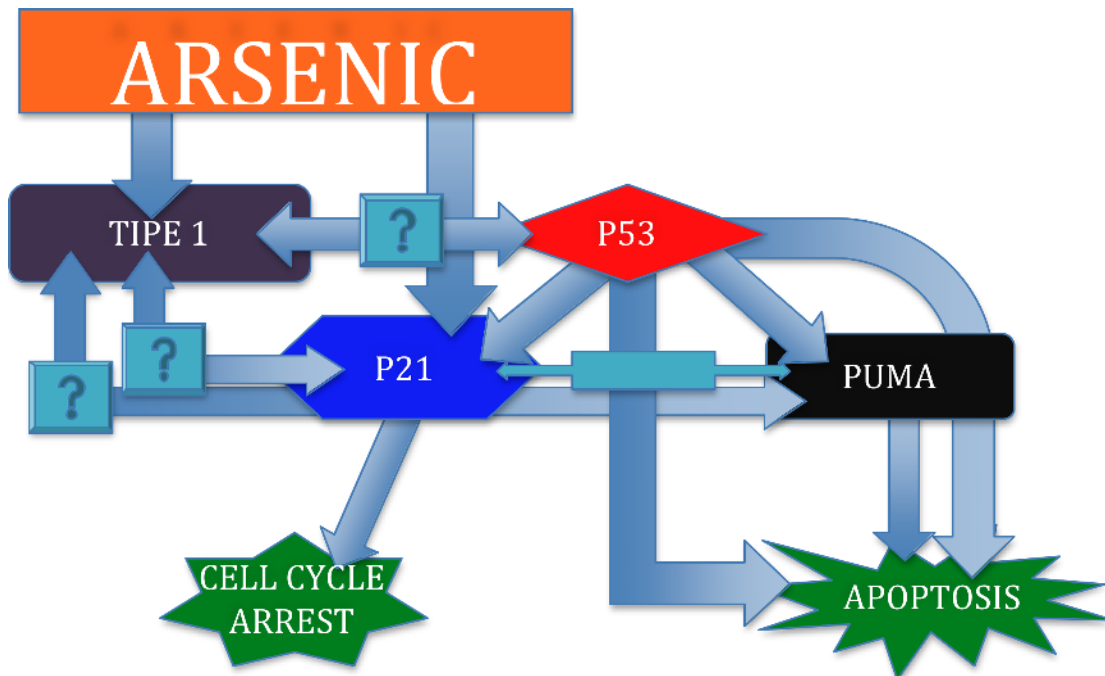


Figure 2: Schematic of interactions between studied apoptotic genes and proposed interactions with *TIPE1*.

Zebrafish were also exposed to butyric acid in an attempt to induce the expression of *p21* to simulate the induction caused by arsenic exposure. Butyric acid has been demonstrated to induce the expression of *p21* and is cheap and readily available, making it attractive as a substitute for arsenic exposure to result an induction of *p21* in zebrafish (14, 22, 37). Upon collection of zebrafish samples, RNA was extracted, converted into cDNA. The resulting cDNA was tested for the expression of target genes and evaluated using quantitative real-time polymerase chain reactions.

A respiratory burst assay of fish exposed to butyric acid indicated reduced production of reactive oxygen species (as seen in the results of a 1mM butyric acid exposure test in figure 11). Additionally, butyric acid-exposed fish had reduced numbers of MPO-GFP phagocytes assessed by flow cytometry.

The next part of our study was a more targeted approach to dissect *p21* pathways involved in apoptosis and immunity using morpholinos. Morpholinos (MO) are

sequences of analog oligomers designed specifically to block the expression of selected genetic sequences. MOs can be customized to silence the expression of virtually any gene of interest.

Using a *p53* MO, we were able to block the expression of *p53*, and then measured the subsequent expression levels of *p21* using qRT-PCR. A decrease in the expression of *p21* was observed, supporting the statement that *p21* is a *p53* target. To better characterize the relationship between *p21* and *p53*, we injected a *p21* MO and measured *p53* expression. This resulted in an increase of *p53*, as well as an increase of *PUMA*, which is downstream of *p53* and separate, but not completely distinct from, the *p21* pathway (15, 29). This suggested a lack of reverse interaction between *p21* and *p53* as it pertains to the butyric acid tests, therefore, the most likely effect of butyric acid is on either *p21* only, *p53*, or both. The increase in *p53* following induction of *p21* is probably not due to a reverse feedback loop directly from *p21* and may actually be due to *PUMA*. In addition to measuring *p53* expression levels as a result of *p21* knockdown with the *p21* MO, the level of reactive oxygen species was also investigated. A respiratory burst assay of the samples collected after the injection of *p21* MO showed a decrease in reactive oxygen species, which indicates that *p21* levels affect the immune response of zebrafish.

While studies of *p21* and *p53* were proceeding, another group in Dr. Kim's lab was working with *TIPE1*. *Tnfap8L1* ("*TIPE1*") is a poorly studied gene that belongs to the *tnfaip8* family (e.g. *TIPE1*, *TIPE2*, *TIPE3*, *tnfaip8*, etc.). Based on numerous studies of *TIPE2* and *tnfaip8*, it is assumed that the largely uncharacterized *TIPE1* protein plays a role in cancer, immunity, and apoptosis (28, 30, 31). Given the potential connection between the work with *p21/p53* and *TIPE1*, these studies followed parallel paths and

merged in some instances. Essential questions that we wanted to answer were: 1) Does the *p21* pathway regulate *TlPE1* expression? 2) Does *TlPE1* regulate the *p21* pathway?

Using the *p21* MO brought about a decrease in *TlPE1*. A decrease in *TlPE1* gene expression was also measured when a *p53* MO was used to silence *p53*. When *TlPE1* MO was used to silence *TlPE1*, increases in *p21*, *p53*, and *PUMA* were observed. These preliminary data leads us to believe that *TlPE1* undoubtedly plays a role in apoptosis.

II. LITERATURE REVIEW

Zebrafish as a Model Organism

The zebrafish has been extensively used as a model organism in genetic studies all over the world. It has shown promise as an effective model for genetic studies that relate to human disease and dysfunctions (16, 20, 23). It is a convenient model to use for a number of reasons. Zebrafish are relatively easy to maintain in aquarium tanks. They exhibit external fertilization and each pair can generate between 100 and 200 fertilized embryos in a single reproductive event. This is highly convenient because the embryos can be genetically modified quickly, and larvae develop in mere days after fertilization. The chorions surrounding the zebrafish embryos are clear, enabling accurate injection and contributing to the visibility of the organism. The zebrafish genome is fully sequenced and annotated, and there are numerous resources and genetic tools available for this model organism.

Arsenic

Arsenic is widely known as a heavy metal toxicant. It is naturally (and sometimes as a result of human causes) found throughout the Northeastern U.S. and is often present in low concentrations in drinking water. According to the U.S. Environmental Protection Agency and the World Health Organization, an acceptable level of arsenic in drinking water is 0.01mg/L (equivalent to 0.1µg/ml). Although this is a very low concentration, even low levels of arsenic are thought to result in negative health effects (8, 19, 24).

Below is a figure demonstrating the presence of arsenic in New England.

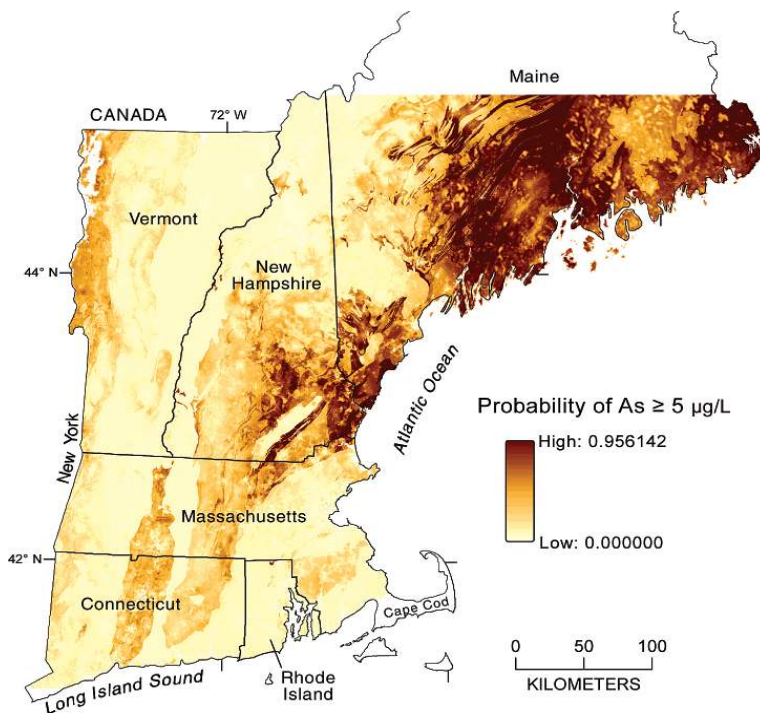


Figure 3. Modified from Ayotte et al. Environ. Sci. Technol. 2006, 40, 3578-3585 (1). Diagram depicting generally high concentrations of arsenic present in waters of New England.

Arsenic has been linked to a wide variety of negative health consequences upon consumption and exposure including reduced immune function, diabetes, and cancer (8, 19, 24). Where apoptosis is thought to help prevent and contain cancerous cells, any link

found between arsenic and genes regulating apoptosis is worth investigating. Arsenic has been demonstrated to increase expression levels of *p21* beyond this study as well (18, 21).

Apoptosis

Apoptosis (programmed cell death) is an important regulatory process. This process ensures proper cell development, and directly eliminates any cell that may develop genetic defects or may otherwise be dysfunctional. Apoptosis is a normal cellular process, and can lead to excessive proliferation of cells with genetic errors if disrupted. Many cancers have been linked to errors in apoptotic pathways that regulate cellular proliferation due to decreased apoptotic processes (4, 13). In addition, an abnormal increase in apoptotic processes can potentially lead to numerous negative consequences, such as muscular atrophy (5). Genetic processes that regulate apoptosis are very intricate and complex interaction pathways. It has been shown that *p21*, *p53*, *PUMA*, and the *tnfaip8* family of genes each play a role in the regulation of apoptosis (9, 13, 15).

P21

P21 is a protein that is the product *cdkn1a* (cyclin-dependent kinase inhibitor 1a). P21 is well known for its role in cell-cycle regulation, cell-cycle arrest and effects on apoptosis. Studies indicate that the upregulation and over expression of *p21* typically leads to cell cycle arrest, and that the protein product of *p21* is a crucial governing

mechanism for regulation a cell's entry into the cell cycle (9, 13, 25). Induction of *p21* is thought to slow the progression of a cell through the cell cycle. This is important in the cells of any organism and especially in which replication is about to occur. Regarding apoptosis, in some situations *p21* is thought to induce apoptosis as its expression increases, but it is typically understood to play a role in 'steering' a cell towards cell cycle arrest rather than apoptosis (9, 13, 25). Most notably, it is thought to regulate a cell's entry to the G2 and M checkpoints (35). *P21* is also important in its regulation of the overall quality of DNA and has been thought in some cases to prevent accumulation of genetic errors in DNA (33).

P21 has a function in cancer cells, but the exact role is somewhat unclear. In a study involving mice it was found that tumor cells were decreased with a decrease in the expression of *p21* (34). Contrary to this study, however, it has also been determined that with a decrease in *p21* expression came increased outbreaks of cancerous cells in mice (2). Variations in *p21* levels alter the likelihood of cancerous growths, but this is a very complex process (13). It has been demonstrated that *p21* feedback loops are highly conserved between mice and humans, promising other vertebrates beyond humans (such as zebrafish) as potentially useful organisms that can be used to study the effects of *p21* (25). To tie back into the main theme, arsenic has been demonstrated to increase expression levels of *p21*, making this gene attractive as a research target (18, 21).

p53 and PUMA

p53 is a pro-apoptotic gene that, upon induction, can either halt the development of a cell or lead to its apoptosis. Of particular interest to this study is the fact that *p53* governs the expression levels of *p21* as well as the apoptotic gene *PUMA* (6, 10, 26). *PUMA* stands for "p53 up-regulated modulator of apoptosis" (15). This gene is downstream from *p53*, and induction is directly correlated with increased apoptotic activity (15). A balance and interaction of some kind between *PUMA* and *p21* is thought to determine whether or not a cell will go through apoptosis or cell cycle arrest; alterations in *PUMA* levels have been implicated in some cancer studies (13). *p53* is thought also to work as a regulator of the cell cycle, acting through its role at the G2 cell cycle checkpoint (6).

TIP1

TIP1 is a gene that is not very well understood. It is known to belong to the tnfaip8 family of genes that has been demonstrated to play roles in diabetes, immune function, cancer, and apoptosis (28, 30, 31). An assumed function of *TIP1*, therefore, is that it, too, has some role in apoptotic pathways. Since little is known about *TIP1* and it has distinct connections to the human tnfaip8 family, this gene is intriguing and attractive as a target for experimentation (28, 30). A central question in my project involves the role that *TIP1* may play in the interplay between apoptotic genes such as *p21*, *PUMA*, and *p53*. Additionally, *TIP1* has been demonstrated to be down-regulated upon arsenic exposure after an analysis of the RNAseq data acquired by members of the Kim Lab

depicting expression levels of hundreds of genes after exposure to arsenic. Dr. Sullivan suggested that characterization of this gene would be worthwhile, and with his help I integrated the examination and assessment of this gene into my project.

Butyric Acid

Butyric acid (which may also be referred to as 'butyrate' in some literature) is a short-chain fatty acid that is commonly found as a by-product of the fermentation and aging of dairy products. Numerous studies have demonstrated this fatty acid to have direct effects on genetic activity. Of particular interest to the Kim Lab is that butyric acid has been shown to induce the expression of *p21* through its inhibition of histone deacetylases (HDAC) (14, 22, 37). A proposed mechanism by which butyric acid induces *p21* expression is through histone hyperacetylation that is a result of the inhibition of HDAC activity by butyric acid. The acetylation of histones in the *p21* promoter region encourages transcription of *p21*. This can result in increased levels of *p21* expression (see figure 4).

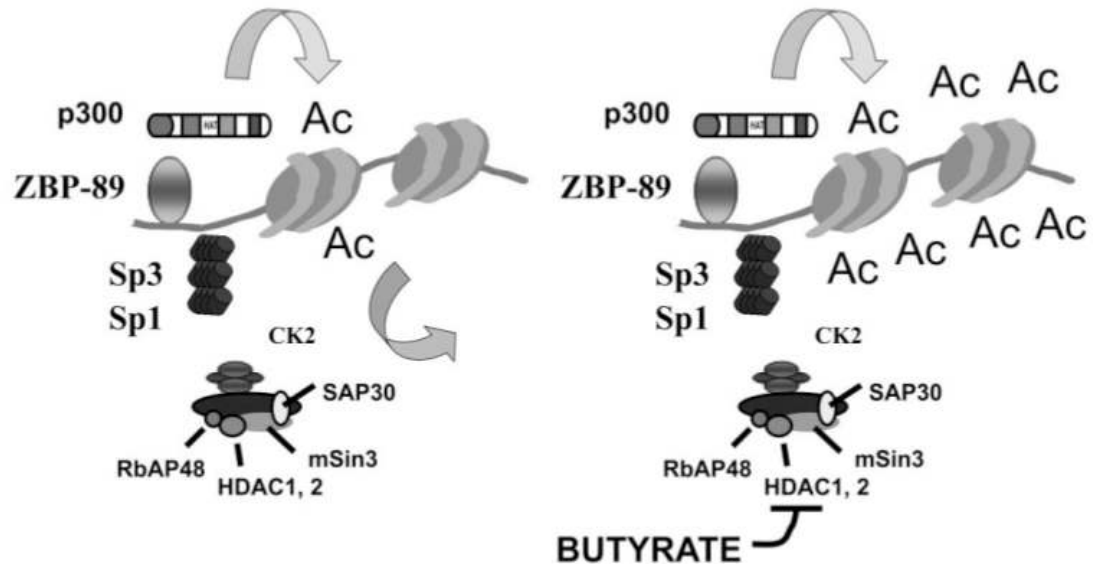


Figure 4: Image taken from Davie, J.R. (2003) (11). *p21* is increased as a result of histone hyperacetylation that results from HDAC inhibition by butyric acid ("butyrate").

III. MATERIALS AND METHODS

Zebrafish Care and Maintenance

Zebrafish were housed at the University of Maine Zebrafish Facility and maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) standards of the University of Maine. Zebrafish strains used throughout these studies include wild-type AB and *Tg (mpx:GFP)*. Embryos were stored at 28°C in egg water (60 ug/ml instant ocean stock salts in distilled water). Egg water was changed daily.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed by using the CFX96 Real-Time PCR Detection System. Gene specific primers for qRT-PCR were designed to

generate single gene-specific amplicons of 125-200 nucleotides. The primers generated are described in table 1. Reactions were performed in the CFX96 Real-Time PCR Detection System according to the manufacturer's instructions. Reactions were performed in a 10uL comprised of 4.8uL of corresponding cDNA, 5.0uL of Evagreen or Quanta Supermix and 0.2uL of each primer. Values of each sample were normalized to the corresponding beta-actin value to determine relative copy number. Numbers of biological replicates ranged from 3 to 5, and technical duplicates were standard procedure.

The relative expression levels of *TIPE1*, *p21*, *p53*, b-actin, and *PUMA* were tested using cDNA of interest (e.g. cDNA from samples injected with *p21* MO) with primers designed for each of these genes. The housekeeping gene used was beta-actin. Below is a table with the sequence used for each primer (see table 1).

Table 1: Primer sequences used in qRT-PCR to test expression levels and fold change of genes in cDNA samples.

Primer	Gene	Primer Sequence (5' to 3')
<i>p21</i>	F	CGGGTTTACAGTTTCAGCTCTTGC
<i>p21</i>	R	ATTACCGAGTGAACGTAGGATCCGC
<i>p53</i>	F	CCCATCCTCACAATCATCACT
<i>p53</i>	R	CACGCACCTCAAAGACCTC
<i>TIPE1</i>	F	GGCGCTCAGACCCAACGCAT
<i>TIPE1</i>	R	GCGCCTGCAGGGCTAGGTTC
b-actin	F	CTAATACACAGCCATGGATGACG
b-actin	R	AAAACCGGCTTTGCACAT
<i>PUMA</i>	F	GGACATCACGATTCTGGGATAA
<i>PUMA</i>	R	GTCCACTCTGCTTTCATCT

Flow Cytometry

Flow cytometry was carried out using mpx:GFP strains of zebrafish present in the zebrafish facility at the University of Maine. Fish samples were raised to 3 days old and

anesthetized in tricaine solution in accordance with IACUC protocol. Five embryos from each sample were placed in 3mL PBS solution, mechanically dissociated and passed through a 40 micron strainer. Samples were transferred to centrifuge tubes, centrifuged for 5 minutes at 1500rpm at 4°C, supernatant was removed. Samples were resuspended in 300µL PBS containing propidium iodide. The sample tubes were then inserted into a BD LSR II (manufacturer) flow cytometer and measured for fluorescence in cells. Between 20000 and 30000 live singlets were counted and number of GFP+ cells were determined within these samples.

Respiratory Burst Assay

Respiratory burst assays were conducted using samples of 1 fish per well. Fish embryos used were treated with one treatment from the following based on the experiment: control MO, *p21* MO, *TIPE1* MO, or 1mM butyric acid. Fish samples were placed into wells with 100µL of egg water, supplemented with 100µL of dihydrodichlorofluorescein diacetate (H2DCFDA), covered with aluminum foil, and incubated at 28°C for 3 hours. Upon oxidation, H2DCFDA becomes fluorescent dichlorofluorescein (24). Subsequent fluorescence was measured which corresponded to level of reactive oxygen species present in each sample.

Morpholino Knockdown

Gene knockdowns were achieved via morpholino antisense oligonucleotides (MOs) against certain zebrafish genes. Morpholino microinjections were carried out in yolks of one-cell stage zebrafish embryos. Morpholino content used depended on the specific sample. Each morpholino solution injected consisted of 8ng of MO mixture with 0.05% phenol red and 1x danieau buffer. The following sequences were used:

Table 2: Morpholino sequences and corresponding quantities used in genetic manipulations of zebrafish embryos

MO Gene	MO Sequence	MO quantity injected
<i>p21</i>	ACAATCTGCAAACACAAACCACAGT	8ng
<i>p53</i>	GCGCCATTGCTTTGCAAGAATTG	8ng
	*A series of two separate morpholinos were used to block <i>TIPE1</i> *	1) 4ng 2) 4ng
<i>TIPE1</i>	1) GTGCTGAACGAGTCCATGATGTCTC 2) CCCCTATTTGAACCGGATACAAGGC	

RNA Extractions and Conversion to cDNA

RNA extractions were performed at time points after administration of treatments depending on the experiment after exposure to treatment. RNA was extracted using the Trizol method. RNA was resuspended in either nuclease-free water or Tris-Cl pH 8.0, samples were quantified using nanodrop and quality was determined through gel electrophoresis. Reverse transcription (RT) reactions were performed to convert total RNA into complementary DNA (cDNA) using the iScript protocol and reverse transcriptase.

Delta Delta Ct Statistical Analysis of Raw Data from qRT-PCR

Delta delta Ct method of analysis was used to interpret results followed methods described by Schmittgen and Kenneth (27). Samples were compared using t-test in Microsoft Excel and GraphPad.

IV. RESULTS

The study has been ongoing in the Kim laboratory. Early on, I had access to cDNA extracted from fish exposed to arsenic and RNA sequencing data set depicting thousands of genes and their levels of expression, relative to a control, after exposure to arsenic. My task was to isolate one or more genes that were both significantly altered in expression after arsenic exposure and involved in innate immunity/cell cycle regulation/apoptosis.

Arsenic Exposure Induces *p21*

RNAseq data that was acquired by members of the Kim Lab was examined for significant changes in gene expression as a result of arsenic exposure. *P21* was significantly induced as a result of arsenic exposure: a log₂ fold change induction with a value of 0.900043 was observed. The embryos were exposed to 10ppb arsenic for 96 hours, and the induction of *p21* that resulted was statistically significant. *P21* is an important regulator of cell cycle arrest and apoptosis.

P21 is a single gene that is involved in a very complex web of interactions between proteins and genes that regulate cellular growth arrest and apoptosis. Two other

important genes involved in such processes are *PUMA* and *p53*. Since these genes are also important in apoptosis, we decided to incorporate them into a few of our tests to better understand the relationship of these three genes.

We performed a series of qRT-PCR tests using a *p21* MO. A method commonly used to select efficacy of the *p21* MO is to examine the phenotype of embryos injected. Often, those injected with an effective dose of the *p21* MO exhibit growth deformities ranging from heart deformities to an obviously stunted and curved tail. Additionally, when compared to control embryos, the vast majority of the *p21* MO fish remain in their chorions 3 days post-fertilization, while at this point nearly all control embryos are free-swimming. This was an excellent series of indicators that we used to determine efficacy of the *p21* MO dose that we used.

Once the effective *p21* morpholino dose was determined, RNA was extracted from these fish and a series of quantitative PCR experiments were performed to look into how the knockdown of *p21* affects other apoptotic genes (see figure 5).

p21 Knockdown can Affect Apoptotic Genes

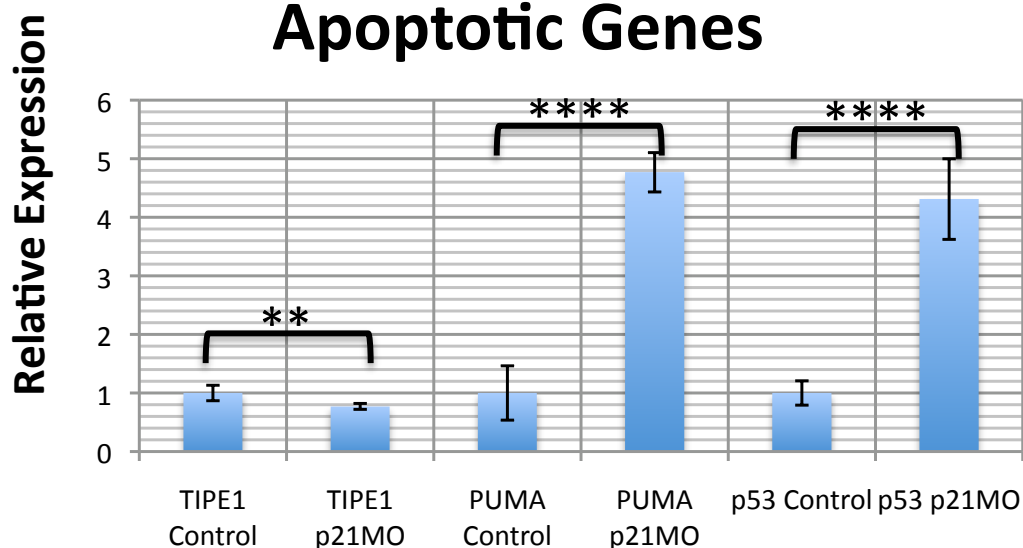


Figure 5. *P21* MO results in differential regulation of *TIPE1*, *PUMA*, and *p53*. $n = 5$, p -values = 0.0066, <0.0001, and <0.0001 respectively. For this and all subsequent graphs/charts and p -values: * = not significant = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

The results demonstrate a slight down-regulation of *TIPE1* as a result of the *p21* MO, and substantial increases in levels of *PUMA* and *p53* expression. It has been cited that *PUMA* and *p21* levels interact to balance each other, and in the event of a decrease of *p21* a cell is more likely to undergo apoptosis as a result of increased *PUMA* levels (13). *P21* knockdown results in an increase of *p53*, and subsequently, we see an increase of *PUMA* expression. *TIPE1* is down-regulated in this instance, indicating that it may play a role as a negative regulator ('inhibitor') of *PUMA*-dependent apoptosis. A hypothetical model of the transcriptional network is shown below in figure 6.

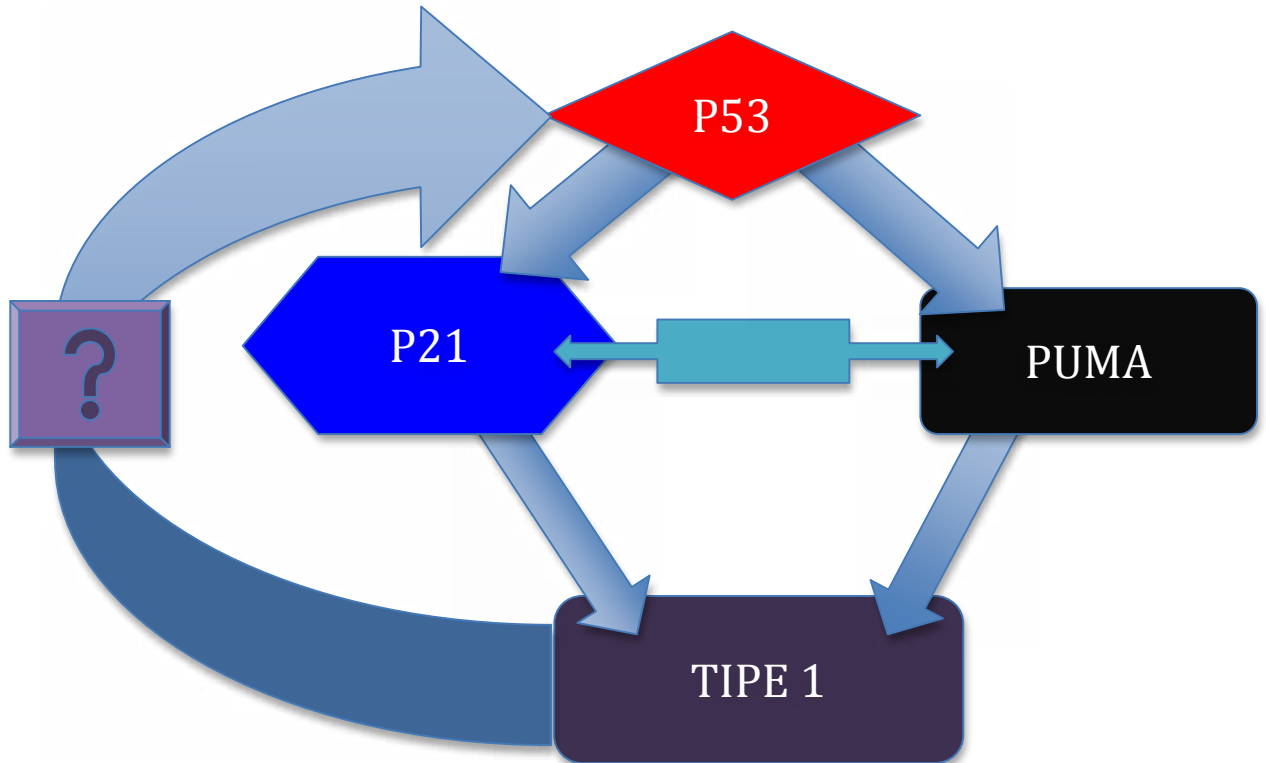


Figure 6. Co-expression network for *p53*, *p21*, *PUMA* and proposed interactions with *TIPE1*.

TIPE1 Involved in Apoptotic Pathways

To look into the effect that *TIPE1* had on apoptotic genes, morpholinos were used to knockdown *TIPE1* expression. A series of qRT-PCR experiments were performed, looking into the expression of genes *p53*, *p21*, and *PUMA*.

TIPE1 Knockdown can Affect Apoptotic Genes

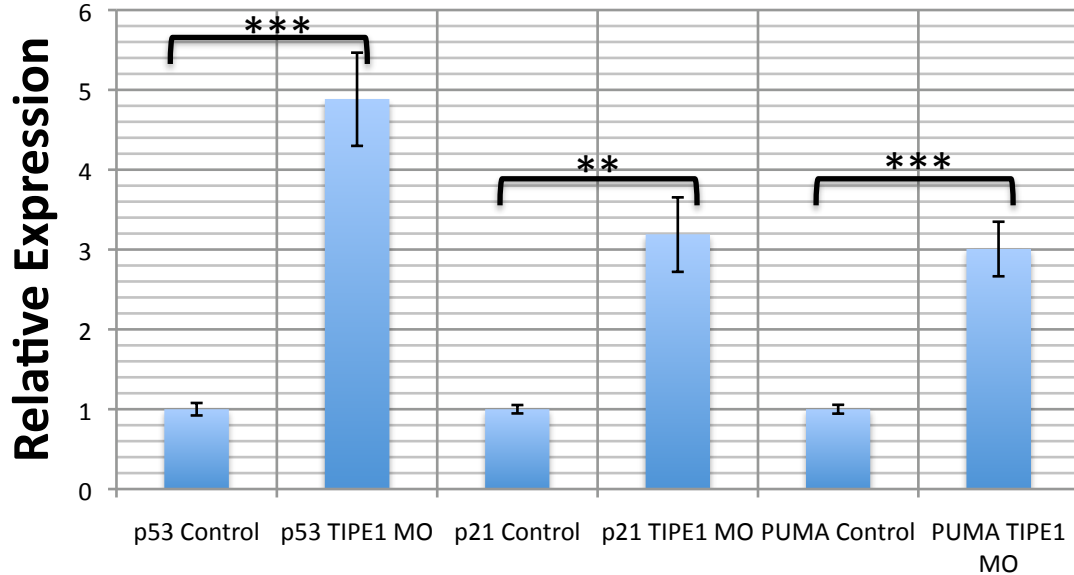
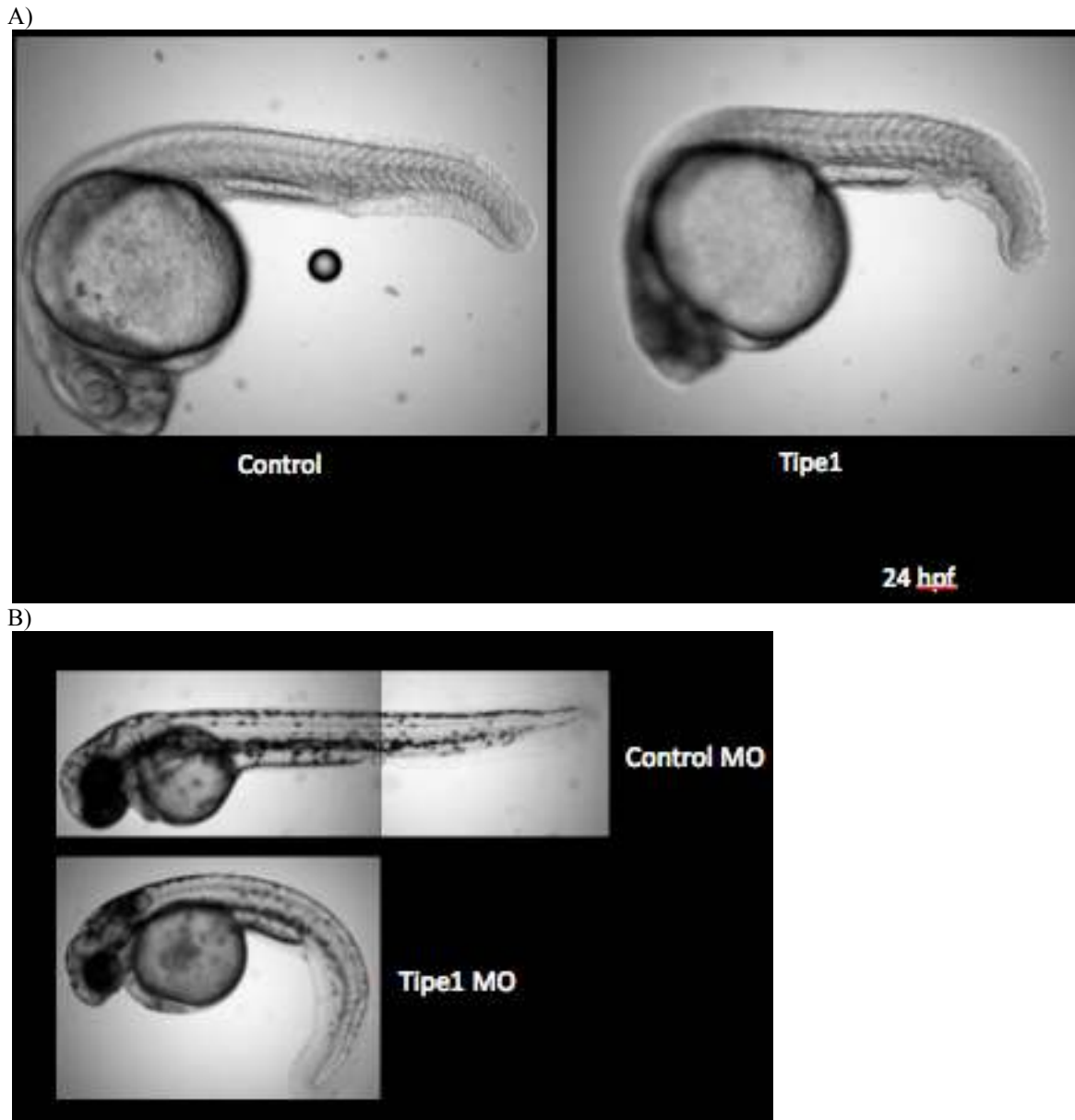


Figure 7. *TIPE1* knockdown results in increased expression of *p53*, *p21*, and *PUMA*. $n = 3$. p -values = 0.0003, 0.0013, and 0.0005 respectively.

The results indicate that a decrease in *TIPE1* expression results in the up-regulation of *p53*, *p21*, and *PUMA*. This is significant in that it further supports the notion that *TIPE1* may be acting as a negative regulator of *p53/PUMA*-dependent apoptosis. This is intriguing, however, in that the decrease of *TIPE1* leads to a decrease in *p21*, while previously a *p21* MO resulted in decreased expression of *TIPE1*. Figures 8A and 8B are images that demonstrate a typical phenotype that results after exposure of an embryo to a *TIPE1* MO. The phenotype below can be used to assure that samples were injected appropriately and the MO is acting as expected.



Figures 8A and 8B: *TPE1* morphants exhibit phenotypic variations from control fish, from Dr. Con Sullivan's departmental seminar presentation (30).

P53 Modulates *TPE1* Expression

Following this round of tests, we moved in a new direction, using morpholinos to knockdown the expression of *p53*. *P53* morpholinos were injected to look into the possible effects that it could have on *p21* and *TPE1* expression.

p53 Knockdown can Affect p21 and TIPE1

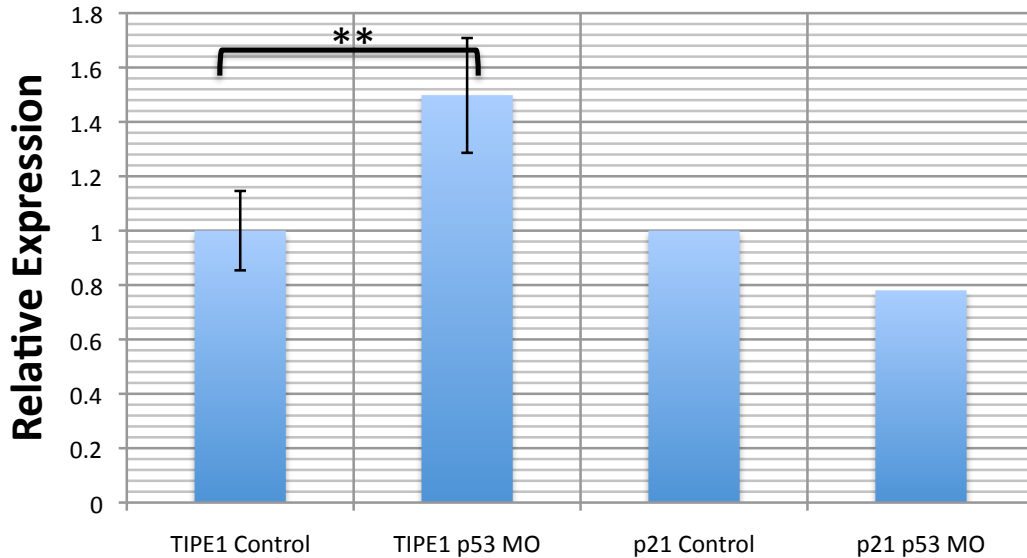


Figure 9. *P53* knockdown decreases expression of *p21* and *TIPE1*. $n = 5$, p -value = 0.0025 for [*TIPE1* control] and [*TIPE1 p53 MO*] data. $n = 1$, p -value = n/a for [*p21* control] and [*p21 p53 MO*] data.

The results showed that, relative to the control, *p21* expression was down-regulated in the *p53 MO* sample. This suggests, as predicted, that a decrease in *p53* leads to a decrease in *p21*. *P21* expression data after *p53 MO* treatment (above) was collected using single replicates of *p53 MO* cDNA and further experiments need to be performed with multiple replicates in order to confirm these preliminary findings. Additionally, relative to the control *TIPE1* expression was increased in *p53 MO* cDNA. This suggests that decreased *p53* acts to increase expression of *TIPE1*; this indicates potential direct interaction between *TIPE1* and *p53*. The number of cDNA samples used in the determination of *TIPE1* expression after treatment with *p53 MO* in this particular experiment was 5. The interaction between *TIPE1* and *p53* is actively being investigated in the Kim lab and studies are ongoing.

Butyric Acid Alters Apoptotic Gene Expression

Butyric acid is a short-chain fatty acid that is a common byproduct formed in the 'spoiling' of dairy products that has been shown to increase the expression of *p21* (22, 37). We attempted to validate this observation, with the goal of developing a reliable method to induce the expression of *p21*. The first step was to determine a concentration of butyric acid to which the embryos would be exposed without lethal effects, and then determine efficacy of butyrate in inducing *p21*. Concentrations higher than 1mM of butyric acid killed all of the embryos, so exposure was carried out 2 days post fertilization and for 24 continuous hours. RNA was extracted from sets of fish that were exposed to varying concentrations of butyric acid.

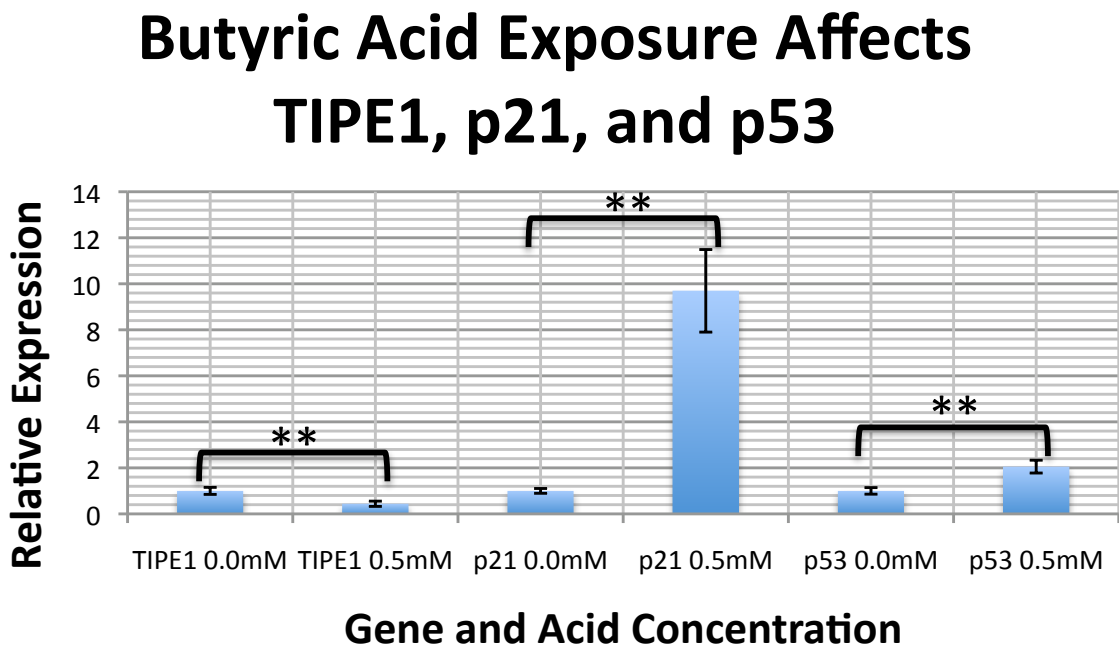


Figure 10. Butyric acid exposure lead to an increase in *p21*, *p53*, and a decrease in *TIPE1*. $n = 3$, p-values for *TIPE1*, *p21*, and *p53* = 0.0070, 0.0011, and 0.0043 respectively.

P21 appears to maintain a constant level of expression until 0.5mM butyric acid, is reached, at which point its expression is greatly increased. In addition, we also observed an induction of *p53* at 0.5mM butyric acid. *P53* is a gene that is upstream of *p21* and has been shown to regulate *p21* (7, 17). This prompted the questions: if butyric acid is inducing *p21*, could *p21* be inducing *p53* expression after butyric acid exposure in conjunction with the *p53* induction of *p21*? Is butyric acid only affecting *p53*, which in turn leads to an increase in *p21*? Is butyric acid affecting both of these genes at the same time? *TIP1* expression appears to decrease as a result of exposure to butyric acid.

Alterations to Apoptotic Genes Leads Respiratory Burst Changes Indicative of Altered Immune Function

To look directly into the innate immune system, assays designed to measure the respiratory burst in zebrafish was employed (see figures 11 and 12).

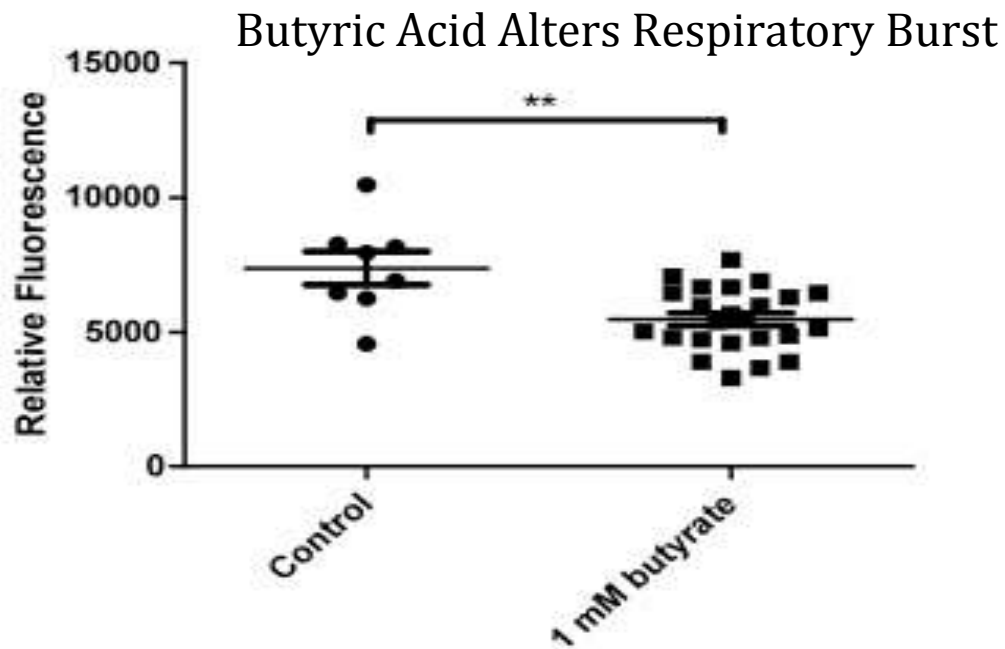


Figure 11. Exposure to 1mM butyric acid decreased respiratory burst. n = 3, p-value = 0.0014.

A decrease in respiratory burst occurred as a result of butyric acid exposure, which is indicative of decreased immune function. A subsequent respiratory burst assay of samples treated with *p21* MO and *TIPE1* MO also resulted in decreased respiratory burst, which is also indicative of decreased immune function (see figure 12).

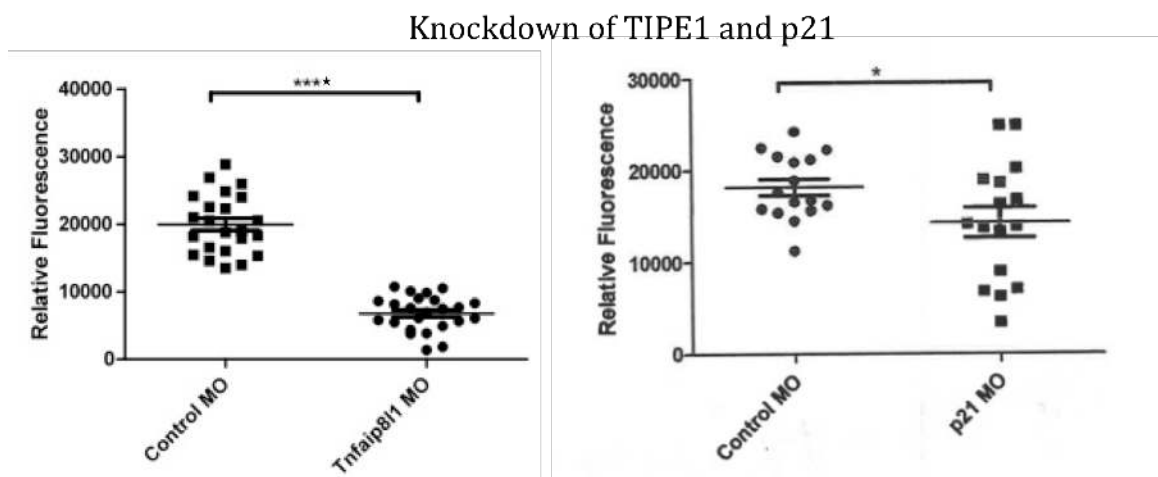


Figure 12. Knockdown of *p21* and *TIPE1* ("Tnfaip81") using morpholinos lead to decreased respiratory burst. n = 3, p-values = <0.0001 and 0.0433 respectively.

In addition, we conducted flow cytometry using fish modified to express GFP in their phagocytes. Using two sample groups, we exposed one group of fish to 0.5mM butyric acid and left another unexposed. An apparent decrease in number of MPO-GFP positive phagocytes (macrophages and neutrophils) was observed in the samples exposed to 0.5mM butyric acid relative to the control (see figure 14). The results from the arsenic exposure tests investigating variations in phagocyte numbers were not statistically significant and therefore no immediate conclusions can be made from the arsenic tests (see figure 13).

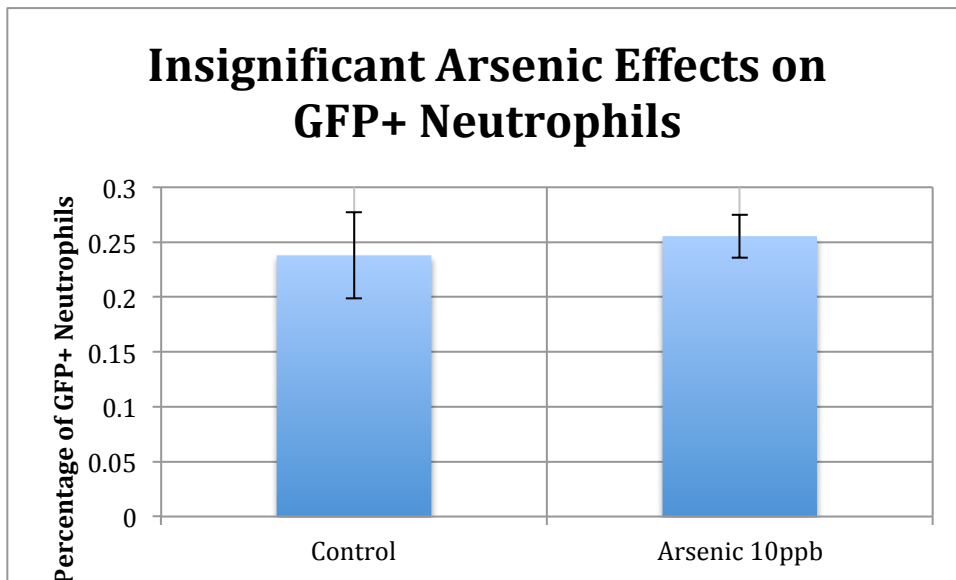


Figure 13: Results of flow cytometer experiment did not show significant change in number of GFP+ phagocytes as a result of 10ppb arsenic exposure. n = 5, p-value = 0.4026 = not significant.

GFP+ Neutrophils Decrease due to Butyric Acid

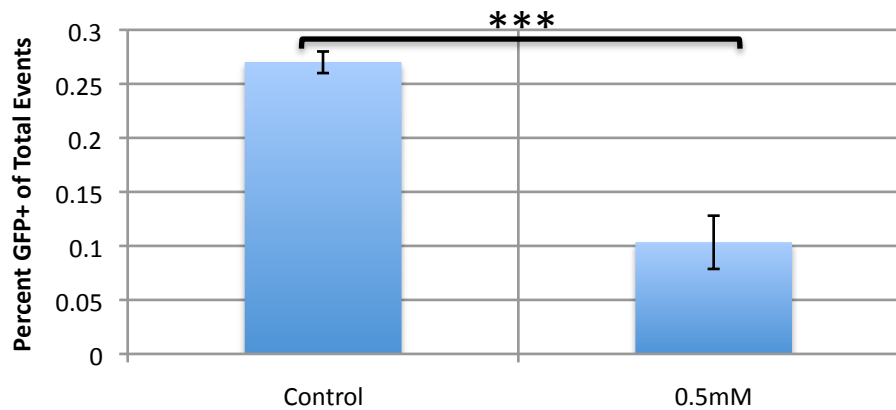


Figure 14: Results of flow cytometer experiment demonstrating decreased GFP+ phagocytes after exposure to 0.5mM butyric acid. n = 3, p-value = 0.0004.

The flow cytometry results for the butyric acid tests are consistent with the results of the respiratory burst assays in that this is likely indicative of depressed immune function in these samples. It appears possible that butyric acid is leading to an increase in apoptosis in cells. There are missing links in this section of the data, including flow cytometry experiments using samples with direct knockdown or induction of *p53*, *TIPE1* and *PUMA* gene expression, as well as a knockdown of *p21*. Samples with knockdowns of genes (such as the use of a *p21* MO, *p53* MO, *TIPE1* MO or *PUMA* MO) could help us to characterize more specifically the effects that butyric acid may be having on these genes.

V. DISCUSSION

P21 is a gene that was identified to be induced upon arsenic exposure in an RNAseq experiment. Further investigation of *p21* and some of the genes it can regulate was performed, and some of these interactions are postulated in figure 15.

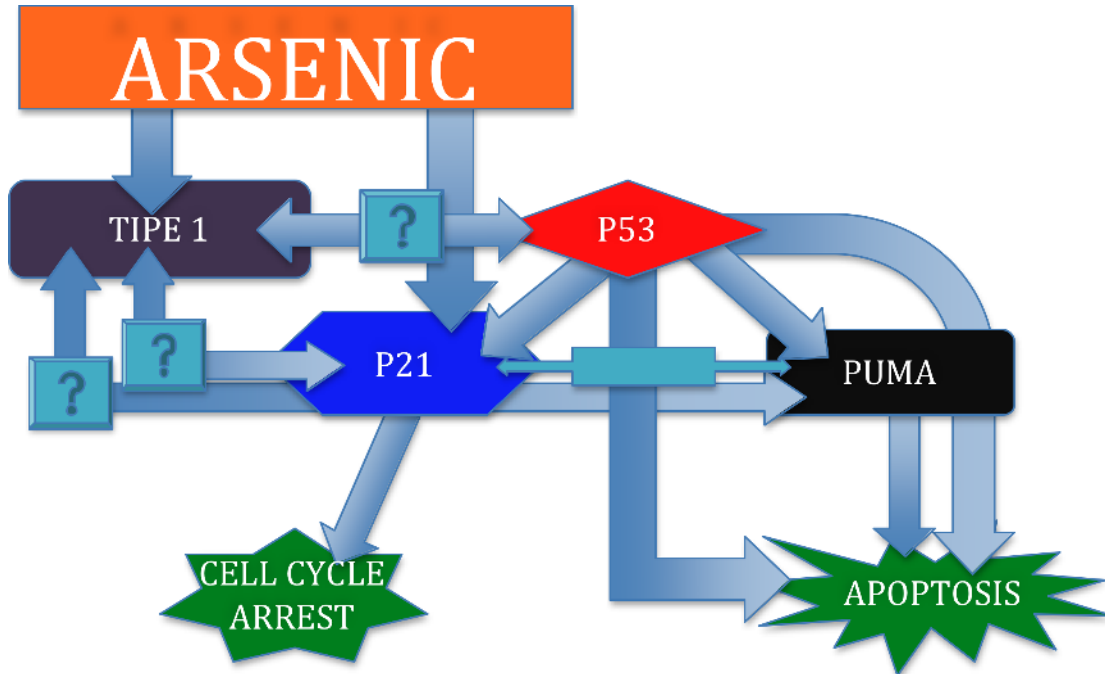


Figure 15: Schematic of interactions between studied apoptotic genes and proposed interactions with *TIPE1*.

P53, *p21*, and *PUMA* all interact to regulate cell cycle and apoptosis, and *TIPE1* is also likely to be interacting with this network (see postulated interaction arrows above in figure 15). Arsenic alters expression levels of *p21* and *TIPE1*, which can alter the balance naturally present in the interactions between these genes.

The long-term significance of this study is yet to be determined, but a few things have been made very clear. Through a series of experiments that either induced or knocked down *p21* and *p53* expression, we have further evidence that *p21*, *p53* and *PUMA* interact with each other to modulate apoptosis. *PUMA* and *p53*, both pro-

apoptotic genes, have been demonstrated to have increased expression upon the knockdown of *p21*. Through induction of *p21* using 0.5mM butyric acid, we also have demonstrated an increase in relative *p53* expression. Similarly, through knockdown of *p53*, we have demonstrated an increase in *p21* expression.

Perhaps the most exciting result involves apparent interactions between the relatively little-known *TIPE1* gene and these three relatively well-characterized genes of *p53*, *p21*, and *PUMA*. Through the knockdown of *TIPE1*, we see a substantial increase in *p53*, *PUMA*, and *p21* gene expression. Additionally, when samples were treated with a *p21* morpholino, a decrease in *TIPE1* was observed. When samples were treated with a *p53* morpholino, an increase in *TIPE1* was observed. With regards to butyric acid, while *p53* and *p21* were induced upon exposure to 0.5mM butyric acid, *TIPE1* exhibited decreased expression. These results are indicative that this gene may play a role in negative regulation of apoptotic pathways.

Additionally, reduced respiratory burst resulted from exposure to 1mM of butyric acid, combined with the knockdown of *TIPE1* and *p21*. The reduced oxidative burst response in fish treated with butyric acid and those with suppressed *p21* and *TIPE1* function shows that these fish may experience a decrease in the functionality of their immune systems. This directly ties into the arsenic-induced decrease of *TIPE1* that was seen in the RNAseq data. Fish treated with butyric acid or *p21* MO both exhibited decreased respiratory burst. Butyric acid is known to induce the expression of *p21*; a decrease in respiratory burst in both the *p21* MO and butyric acid exposed fish may be due to *p21*-independent effects of butyric acid. This respiratory burst assay should be

repeated in the future using 0.5mM butyric acid, which was the concentration used in the experiment from which qRT-PCR data showed a significant increase in *p21*.

The results of flow cytometric analysis need to be expanded, but the data collected suggest a decrease in numbers of MPO-GFP+ phagocytes in fish exposed to 1mM butyric acid. This also suggests some change in immune response (likely to be a general decrease in functionality of the immune system) as a result of butyric acid exposure. This test should also be repeated using 0.5mM butyric acid. Further flow cytometry experiments should be performed with direct modifications to *p21*, *p53*, *PUMA*, *TlPE1*, and after exposure to low levels of arsenic.

As a result of arsenic exposure, *TlPE1* and *p21* were both altered; *TlPE1* expression decreased while *p21* expression increased. Based on the studies that we have performed here, it is clear that *TlPE1* and *p21* both play significant roles in the regulation of apoptotic pathways related to gene expression. The resulting effect is the altered ability of cellular mechanisms to govern proper cellular apoptotic response. The further characterization of *TlPE1* and acquisition of more data may help to uncover the effects of an induction of *p21* by arsenic and will help us gain a better understanding of potential physiological consequences of arsenic exposure.

VI. FUTURE DIRECTIONS

In order to better understand the pathways discussed here, there need to be additional studies to better characterize relationships between *p21*, *p53*, *PUMA* and *TlPE1*. Based on the results from *p53* and *PUMA* studies above that indicate an increase

in apoptosis with elevated levels of these genes, I predict decreased presence of MPO-GFP+ phagocytes in flow cytometry experiments using *p21* MO samples. This is one test that needs to be completed to better characterize how *TIPE1* affects the immune system through *p21*.

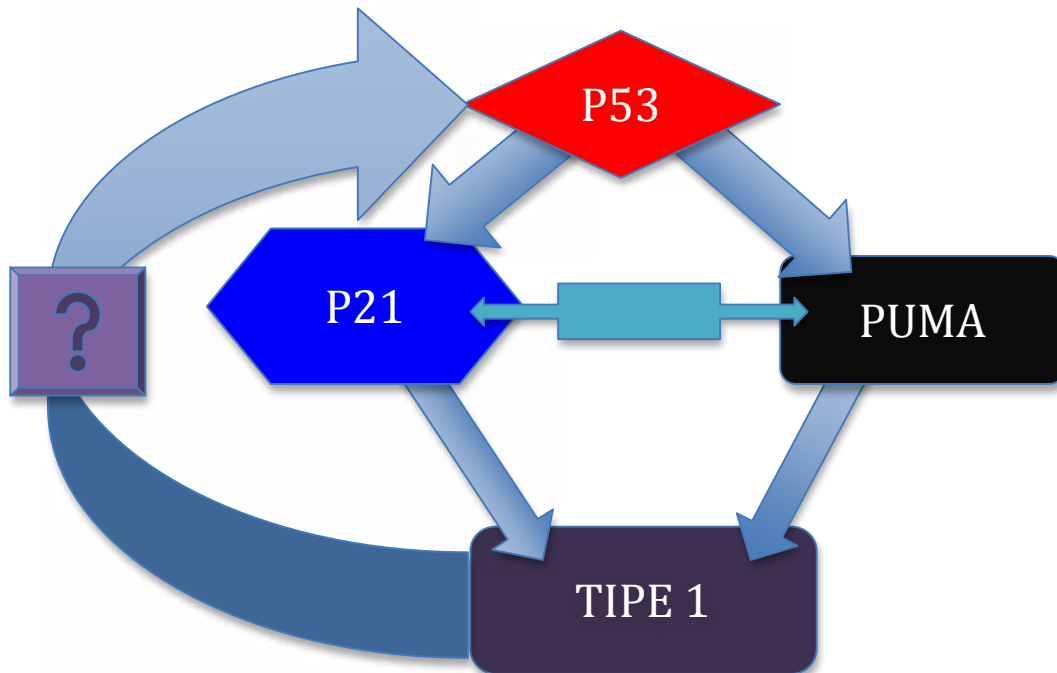


Figure 16. Diagram demonstrating possible interactions between *TIPE1* and *p21*, *p53*, and *PUMA*.

Other future tests that should be performed include:

- *PUMA* MO samples to test for expression levels of *p21*, *p53*, and most importantly *TIPE1*
- 0.5mM butyric acid exposure, followed by qRT-PCR measurements of expression levels of *PUMA*
- 0.5mM butyric acid exposure, followed by respiratory burst assay to be consistent with other butyric acid data collected and used in qRT-PCR experiments

- Combinations of morpholinos targeting *p53*, *p21*, and *PUMA* with subsequent tests of expression levels of *TIPE1*
- Induction of *TIPE1* with tests of subsequent expression levels of *p21*, *p53*, and *PUMA*
- Alterations of *p21*, *p53*, *PUMA*, and *TIPE1* followed by flow cytometer measurements using MPO fish exhibiting GFP on their phagocytes

It is very exciting for me to think about the possibilities surrounding this study. The results that we gathered show promise in the dissection of apoptotic pathways that may be altered and changed as a result of arsenic exposure. Such alterations to natural balances and harmonies that occur in these pathways may lead to negative health consequence such as cancer and decreased immune function. Unraveling the effects of arsenic can help us understand how much we should be concerned with the consequences of exposure to this heavy metal. A better understanding of genes such as *p21*, *p53*, *PUMA*, and especially uncharacterized genes such as *TIPE1* are the first step in understanding the effects arsenic has on animal physiology and subsequent health consequences that we may see in humans. This is also the first step in determining how to prevent and treat disorders that may result from arsenic exposure. Additionally, extensive studies of the interplay that occurs between *p21*, *p53*, *PUMA*, and *TIPE1* may lead to discoveries that can help treat and prevent some of mankind's greatest ailments, such as cancer.

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Author's Biography

Gabriel "Gabe" Vachon was born in Ellsworth, Maine in January of 1991. He grew up in Ellsworth and graduated from Ellsworth High School in 2009, fourth in his class. He attended the University of Maine the following fall, enrolled as a Biology major in the Honors College after receiving the Top Scholar award. Gabe, during his time at the University of Maine, was a Resident Assistant and a member of the University Volunteer Ambulance Corps.

Upon graduation in May of 2013 with a B.S. in Biology and a minor in Chemistry, Gabe plans to begin job shadowing, working, and volunteering in local hospitals and intends to apply to medical school the following fall.