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Micro-RNA mediated regulation of a cytokine factor: TNF-alpha: an exploration of gene expression control in proliferating and quiescent cells

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Thesis

**MICRO-RNA MEDIATED REGULATION OF A CYTOKINE FACTOR:
TNF-ALPHA: AN EXPLORATION OF GENE EXPRESSION
CONTROL IN PROLIFERATING AND QUIESCENT CELLS**

by

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B.S., Emory University, 2010

Submitted in partial fulfillment of the
requirements for the degree of
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ABSTRACT

Two types mechanisms that control gene expression involve cis-regulatory factors and trans-regulatory factors. Cis-acting regulatory RNAs include targeted messenger RNA (mRNA) specificity and AU-rich elements (AREs). AU-rich mRNAs are a subcategory of mRNAs that have AREs in their 3'-Untranslated Regions (UTRs). These ARE-genes have been observed to correlate with rapid mRNA decay patterns. They comprise approximately 12% of all transcripts and are known to encode for a group of proteins that have involvement in the inflammatory response. Trans-acting regulatory mechanisms are micro RNAs (miRNAs) in eukaryotes, and small RNAs (sRNA) in prokaryotes. Misregulation of these mechanisms can lead to many disease states if rapid mRNA decay does not occur, leading to tumorigenesis, and eventually, different types of cancer. In this project, the TNF- α ARE was studied in both serum-positive and quiescent G0 conditions in order to analyze whether the translation of the gene differed in any respect due to the binding of a known miRNA called miR-130a. Additionally, both serum-positive and one-day serum-starved quiescent G0 conditions were analyzed for eIF5B and FXR1 levels to analyze whether there was a correlation between the two proteins.

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LIST OF ABBREVIATIONS

| | |
|-------------------------|--|
| λ N..... | λ bacteriophage antiterminator protein N |
| 3' | 3 prime |
| 5' | 5 prime |
| ARE..... | AU-rich element |
| BJ fibroblasts | Skin fibroblasts |
| <i>C. elegans</i> | <i>Caenorhabditis elegans</i> |
| cDNA | Complementary DNA |
| DNA | Deoxyribonucleic acid |
| DPBS..... | Dulbecco's Phosphate-Buffered Saline |
| EF | Elongation factor |
| eIF5B..... | Eukaryotic initiation factor 5B |
| FBS | Fetal Bovine Serum |
| FXR1 | Fragile X mental retardation |
| G0..... | Quiescence |
| GTPases | Guanosine-5'-triphosphatases |
| IF | Initiation factor |
| HEK293 | human embryonic kidney 293 |
| LGlu | L-Glutamine |
| mRNA..... | Messenger RNA |
| miRNA..... | MicroRNA |

| | |
|-----------------------------|--|
| mL..... | Milliliter (s) |
| NF- κ B | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| PAZ..... | Piwi Argonaute Zwiille |
| P-body..... | Processing body |
| PIWI..... | P-element induced wimpy testis |
| P/S | Penicillin and streptomycin |
| qPCR..... | Quantitative polymerase chain reaction |
| rpm | Revolutions per minute |
| RISC..... | RNA-induced silencing complex |
| RNase | Ribonuclease |
| RBP | RNA binding protein |
| RNA | Ribonucleic acid |
| SDS | Sodium dodecyl sulfate |
| sRNA..... | Small RNA |
| siRNA | Small interfering RNA |
| shRNA..... | Small hairpin RNA |
| TNF- α | Tumor-necrosis factor-alpha |
| THP1 | Human monocytic cell lines |
| tRNA-Met _i | Initiator methionine tRNA |
| uL | Microliters |
| UTR..... | Untranslated region |

INTRODUCTION

1. Post-Transcriptional Gene Regulation

Transcriptional regulation in cells was first discovered approximately fifty years ago in bacterial cells.¹ Since that time, science has made strides in understanding the regulation of gene expression in cells of the human body, which occurs on many levels and is different for each type of cell. Gene expression is controlled by many transcription factors, cofactors, and chromatin regulators. It has also been discovered that there are many other forms of transcriptional regulation that, if misregulated, can lead to tumor formation and other diseases.² Post-transcriptional regulatory mechanisms also exist that can increase or decrease gene expression in cells. In 1993, Victor Ambros, Rosalind Lee, and Rhonda Feinbaum made a discovery as they were studying the *lin-4* gene, which controls the timing of *C. elegans* larval development.³ It was found that when they isolated the *lin-4* gene, a messenger ribonucleic acid (mRNA) (RNA), was not produced, but instead the gene produced a noncoding RNA that was partially complementary to the 3' (3 prime)-untranslated region (UTR) of the *lin-14* mRNA. This partial complementarity of the noncoding RNA to the 3'-UTR led to the inhibition of translation of the *lin-14* mRNA into LIN-14 protein.³ The non-coding RNA was eventually termed microRNA (miRNA). They are small, single-stranded molecules that contain approximately 10 to 25 nucleotides. They often take part in post-transcriptional gene regulation by binding to the 3'-UTR of a molecule of mRNA. MiRNAs generally result in degradation or inhibition of translation, but may also lead to upregulation of translation of a certain protein. Each miRNA may have more than one target, and more than one

miRNA can bind to a single mRNA molecule. To date, approximately two thousand unique miRNAs have been discovered, which seemingly may regulate up to two-thirds of the human genome.⁴ These miRNAs can be found in nearly all bodily fluids, including blood, urine, milk, saliva, and cerebrospinal fluid. It has been shown that miRNAs respond to both intracellular and extracellular signals.^{4,5}

2. Trans- and Cis- Acting Regulatory RNAs: A Brief Overview

Gene expression can be controlled by regulatory RNAs in many different ways. Two such mechanisms are trans-acting regulatory mechanisms and cis-acting regulatory mechanisms. Two examples of trans-acting regulatory mechanisms are miRNAs in eukaryotes, and small RNAs (sRNA) in prokaryotes.⁶ sRNAs play a substantial role in bacterial regulation. Most of the trans-acting sRNAs bind to the 5'-UTR or the translation initiation region of specific mRNAs. Moreover, it has been shown that sRNAs contain specific domains and scaffolds that recruit RNA chaperones to aid in gene regulation. According to Sharma et al., antisense sequences from one sRNA can be fused to Hfq, an RNA-binding protein (RBP), of another sRNA to create a hybrid sRNA.⁷ This binding allows bacteria to quickly adapt to their surroundings by upregulating or downregulating translation as appropriate. In order to repress translation, the sRNA competes with the 30S ribosome for mRNA binding. Since translation initiation occurs at the Shine-Dalgarno sequence, sRNAs often bind to this region, thereby inhibiting translation from occurring.⁸

MiRNAs in eukaryotes have a different method of regulating gene expression. They are usually produced from transcripts that have stem-loop structures. They are then

processed in the nucleus by Ribonuclease (RNase) III enzyme Drosha, and DGCR8 in mammals. Then, cleavage occurs, and a pre-miRNA is exported into the cytoplasm, where the RNase III endonuclease Dicer complex processes the pre-miRNA into a final miRNA. This is coupled with the miRNA being assembled into a RNA-induced silencing complex (RISC), which is the effector of RNAi.⁸ The most vital part of the RISC complex is a protein called Argonaute (AGO). They have been found in RISC complexes across a spectrum of various organisms. The AGO protein family is diverse, with all members containing a Piwi Argonaute Zwiile (PAZ) domain, which is involved in miRNA/small interfering RNA (siRNA) binding, and a P-element induced wimpy testis (PIWI) domain, which is related to RNaseH endonucleases and functions in slicer activity. In humans, a variant called AGO2 is the only one that has endonuclease activity.⁹ AGO2 is bound by GW182, a processing body (P-body), in normal proliferating cells. GW182, in turn, recruits other factors and coordinates downstream activities. It has been studied and elucidated that the N-terminus of the GW proteins has many GW repeats, which is a requirement for the binding of AGO proteins. G is the symbol for the amino acid glycine and W is the symbol for the amino acid tryptophan. This binding between the two proteins AGO2 and GW182 are said to be “AGO hooks.”¹⁰

The cis-acting regulatory RNAs include targeted mRNA specificity and AU-rich elements (AREs). To expand upon targeted mRNA specificity, UTRs of transcripts often have polyadenylated regions that can prevent or allow miRNA activity. Other factors also come into play at the UTR regions and can be adjusted and altered for each tissue, creating a very specific mechanism.⁴ Yet another type of cis-acting regulation of gene

expression are AREs. AU-rich mRNAs are a subcategory of mRNAs that have AREs in their 3'-UTRs. They comprise approximately 12% of all transcripts and are known to encode for a group of proteins that have involvement in the inflammatory response, immune response, transcription, proliferation, RNA metabolism, development, and signaling. These ARE-genes have been observed to correlate with rapid mRNA decay patterns.¹¹ As can be noted, misregulation of this mechanism can lead to many disease states if rapid mRNA decay does not occur, leading to tumorigenesis, and eventually, different types of cancer.

3. Quiescence: Activation of mRNAs

Quiescence (G0) is a phase in which a subset of dividing cells escape from harsh, or unsuitable conditions and enter a state of arrest until the conditions are more stable for the cell. This allows the G0 cells to avoid a destructive state that may not be reversible. During the G0 state, cells can still carry out functions that allow them to mature, translate proteins that are needed as regulatory factors, and develop the appropriate machinery needed for harsher conditions.¹² These cells often still need certain genes to be expressed in order to carry out several functions. They need these proteins to resist harsh conditions and can be achieved through post-transcriptional mechanisms that may or may not include upregulation by miRNAs.¹³ Translational activation can occur through RBPs, or through micro-RNP-associated factors.¹⁴ G0 can also occur by cell-to-cell contact of high density. Many studies have been conducted that show that miRNAs are important regulators in the G0 cell.⁴ MiRNAs usually target 3'-UTRs. The length is often modified by mechanisms such as alternative splicing or alternative cleavage and polyadenylation.

Shortening of 3'-UTRs has been shown to coincide with proliferation in many types of cells, which can lead to aberrant growth in cancer cells.¹⁵ In G₀, the 3'-UTRs are longer; therefore, they are more subject to miRNA regulation. As outlined above, in normal proliferating cells, AGO2 is bound by GW182 during translation. In the G₀ state, however, it has been shown by Vasudevan and Steitz that AGO2 is instead bound to fragile X mental retardation (FXR1) protein to enhance translation.¹⁶ In their experiment, they studied the tumor necrosis factor-alpha (TNF- α) gene that is activated in serum-starved G₀ cells. In particular, they studied the ARE of TNF- α , which regulates both translation and mRNA stability for the gene by recruiting certain proteins. They conducted experiments that confirmed that AGO2 has a direct serum-starved role in translation. They bound AGO2 to a luciferase reporter, using a λ bacteriophage antiterminator protein N (λ N) peptide. This experiment showed a fivefold upregulation of translation efficiency.¹⁶ Another experiment that Vasudevan and Steitz performed allowed them to confirm that FXR1 was recruited and bound to AGO2 in order to function as a translation activator. They showed that by knocking down 75% of FXR1, a loss of translation activation in serum-starved conditions occurred. This indicated that FXR1 and AGO2 were bound together and needed for translation activation to occur in these conditions or that FXR1 may be needed in a downstream step of translation.¹⁶

4. The Relevance of Quiescence to Clinical Aberrations: Cancer

The progression of cancer is a very complex and complicated process. Many mutations need to occur, which can lead to various “hallmarks” of cancer. Such hallmarks of a cancer cell include eluding the process of apoptosis whereby a cell can

induce self-death, growth signals become self-sufficient, antigrowth signals become fruitless, cells acquire invasive and metastatic capabilities, DNA replication increases in rate, and angiogenesis (vessel development and growth) occurs in order for the cell to get the right amount of oxygen and nutrients.

Adult stem cells are usually the cells that are able to replicate without any limit. It is believed amongst cancer researchers that transformed stem cells may be the cause of many cancers. A G0 state is suggested to be important for many of these adult stem cells in order to maintain a pool. This state is needed for cells to have the ability to maintain their proliferative capacity. Moreover, G0 is needed for stem cells to inhibit differentiation and also to limit accumulation of mutations during the process of deoxyribonucleic acid (DNA) synthesis.

The relevance of the G0 state to medical and clinical interventions is that while chemotherapy can kill rapidly dividing cells, G0 cells are often resistant to conventional chemotherapy.¹⁷ These cells that survive during the medical course can thereby enter the cell cycle repeatedly and restart a tumor state, termed recurrence. This process can be repeated multiple times. Hence, a course of chemotherapy that may be prescribed to a patient may be ineffective. There is not enough research that identifies and explains exactly how G0 contributes to cancer stem cell biology. In order to uncover the mystery, it is important to study G0 and its mechanisms.¹⁸

One such experiment provided relevant indication that G0 played a role in cancer stem cells. Dembinski and Krauss studied cancer stem cells in great depth through the use of Vybrant Dil cell-labeling solution by Life Technologies.¹⁹ Dil is a lipophilic

membrane stain that diffuses across the cell membrane and stains the entire cell. It is orange-red in color and similar to the compound tetramethylrhodamine. This method was used to label adenocarcinoma cells of the pancreas. Then, flow cytometry was conducted to sort the labeled cells. Dil labeling allowed for the retention of slow-cycling cells, which were identified as Dil⁺/SCCs. They consisted of approximately 3% of all the cells that were sorted through the flow cytometer. These cells also showed an elongated fibroblast shape and an increased ability to transition between epithelial and mesenchymal states. Fibroblast-like cancer stem cells often have stem cell properties. Moreover, the sorted Dil⁺ cells showed a two and a half to tenfold increase in soft agar colony forming ability, a twofold ability to invade, and more than a tenfold ability in xenograft formation in comparison to the non-labeled cells.^{19, 20}

5. MicroRNAs interact with AREs; Exploration of miR-130a interacting with TNF- α ARE

As previously mentioned, a cis-acting type of regulation of gene expression involves AREs. ARE-genes are known to have rapid mRNA decay patterns, and if misregulated, can lead to disease states such as cancer. In most human cell lines, the TNF- α ARE enhances translation relative to mRNA levels if the cell is serum-starved. Serum-starvation ultimately leads to cell-cycle arrest and replication ceases. TNF- α is a cytokine, which is usually stimulated in lymphocytes.²¹ Monocytes that circulate in the blood can eventually adhere to inflamed tissues or even if a tumor is penetrating in a certain tissue. This leads to growth arrest in these cells and cytokine expression is concurrently increased. TNF- α is one of the cytokines that are upregulated. In turn, TNF-

α can upregulate other cytokines that are needed for macrophages to mature.²² This whole process can be replicated in cell culture if the cells are serum-starved.

In an experiment done by Zhang et al., they found that a specific miRNA, miR-130a, regulated TNF- α levels by binding to the ARE region of the gene.²³ In their study, they found that nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and miR-130a promoted cervical cancer to grow by increasing cell number. They revealed that miR-130a targets the 3'-UTR of TNF- α , thereby repressing the TNF- α gene to be translated. Low TNF- α levels then activates NF- κ B to upregulate miR-130a to be expressed. A feedback signaling pathway exists that regulates this growth and interplay between NF- κ B, TNF- α , and miR-130a.²³ This study and many others show that AU-rich elements play an important part in regulating translation of specific genes.²³

6. The Protein eukaryotic initiation factor 5B (eIF5B): An Overview of its Role in Translation

Translation is the process whereby the message encoded by an mRNA transcript is translated into a protein. Translation initiation is a complex process in eukaryotes, as can be seen by how many initiation factors (IFs) are needed to get the process of translation started. Approximately twelve initiation factors are needed in eukaryotes, whereas only three are needed in bacterial cells.²⁴ The elongation factors (EF) EF-Tu and EF-G, along with the initiation factors eIF5B, aIF5B, and IF2, are known as the canonical translational Guanosine-5'-triphosphatases (GTPases), and are needed for cellular function. This indicates a strong belief that the function of these elongation and initiation factors was secured at an early stage of evolution. Still, many questions remain about the

active and inactive states of these proteins that are involved in subunit joining. Moreover, their functions differ depending on the three domains of life - Archaea, Bacteria, and Eukarya.²⁴

In eukaryotes, translation initiation is a multifaceted process. Many parts join together to carry out this function. The initiator methionine tRNA (tRNA-Met_i), 40S and 60S ribosomal subunits are assembled together by many eukaryotic initiation factors and eventually form an 80S ribosome. The protein eIF5B is needed in order to join the 40S and 60S ribosomal subunits during translation and functions as a translational GTPase. It is considered an analog of IF2, which is present in bacterial cells. The protein eIF5B also has an important role in ribosome proofing during the pre-40S stages. Yet, another role of eIF5B involves the stabilization of tRNA-Met_i joining, and is required for the translation of some viral mRNAs.²⁵

7. An Observation of a Direct Link Between eIF5B Levels and the G0 state

It has been observed in human monocytic cell lines (THP1) cells that there is a direct link between eIF5B levels when G0 is induced by serum-starvation.²⁵ One day of serum-starvation is distinct from an elongated time of serum-starvation. There is an overall decrease in global translation, but certain mRNAs are upregulated in order to support the cell so that cell death does not occur. Vasudevan et al. showed that eIF5B levels increase transiently in THP1 cells one day after serum-starvation, but not in skin fibroblasts (BJ fibroblasts).²⁵ They concluded that eIF5B may be important for translation during serum-starvation in only specific cell types and may not be a universal increase in all cell types. They also found that when they knocked down eIF5B in serum-starved

cells using small hairpin RNA (shRNA), proliferation of cells decreased compared to when eIF5B was present. This showed that G0 arrest occurred faster in eIF5B depleted cells, indicating that eIF5B may be a possible inhibitor of G0 by allowing for certain mRNAs to be translated.²⁵

In order to show the opposite, Vasudevan et al.²⁵ conducted experiments in which eIF5B was overexpressed. Their hypothesis was that if eIF5B caused inhibition of G0, then overexpression should block G0 entry completely, and cause cell death instead. Their experiments showed that there was a decrease in cell survival after one day of overexpressing eIF5B in serum-starved conditions. In serum-positive conditions, however, cell death did not occur and proliferation resumed at a similar rate to normal proliferating cells.

Stated above, one of the roles of eIF5B involves stabilization of tRNA-Met_i joining. Vasudevan et al. showed that when eIF5B is immunoprecipitated from cells that are serum-starved for one day, there were increases in eIF5B-tRNA-Met_i complexes.²⁵ There was approximately a one and a half fold increase as compared to serum-positive cells.

AIMS

After analyzing the current research, experiments were conducted in human embryonic kidney 293 (HEK293) cells. Initially, THP1 cells were used for experimentation. There were problems in transfection of these cells, and therefore HEK293 cells were used instead. These cells are derived from a human embryonic kidney cancer cell line. These cells can be transfected easily, which is the reason it was

chosen as the cell of choice for experimentation. In addition to confirming that eIF5B is upregulated in one day serum-starved conditions in HEK293 cells, FXR1 levels were also analyzed using western blot analysis after one day-serum starvation.

Additionally, there is an upstream seed region in front of the TNF- α ARE where miR-130a is known to bind. The ARE region was mutated to check if it affected translation levels of TNF- α protein in serum-positive and serum-starved G0 conditions. Moreover, the seed region was also mutated at a designated nucleotide to check if the ability of miR-130a to bind to the miRNA seed region affected TNF- α levels significantly. Since G0 is an important state in which cells can enter to resist chemotherapy and TNF- α has an important role in the inflammatory process that is involved in cancer tumorigenesis and malignancy, experiments were conducted to check whether there was an increase in TNF- α levels in the G0 state as compared to the serum-positive proliferative state. If these important questions can be answered, possible RNA based chemotherapeutics can be developed accordingly that may alter the gene expression of certain G0 cells that may eventually lead to cancer. MiRNAs have been a recent discovery of gene regulation and expression and their functions are still being investigated.

Mutated and wild-type sequences of the ARE region and the upstream seed region of miR-130a of TNF- α were transfected into HEK293 cells. The cells were harvested and assayed for luciferase activity. RNA was isolated from the cells and then complementary DNA (cDNA) was prepared in order to perform quantitative polymerase chain reaction (qPCR) and quantify the translation efficiency of the cells, which would provide insight

as to whether the TNF- α gene was being translated and the degree to which it was being translated. The methods, primers, plasmid DNA sequences, and materials that were used for these experiments are outlined in the following paragraphs.

For reference, the TNF- α ARE is 34 nucleotides long and is found in the 3'-UTR region of the TNF- α mRNA. AREs usually have many adenine and uracil bases in their mRNA, hence, they are known as AU-rich elements. The following sequence of deoxyribonucleotides, when undergoing transcription, creates an mRNA that is the TNF- α ARE.¹⁶

5' ATTATTTATTATTTATTTATTATTTATTTATTTA 3'

In the experiments that were conducted, the above ARE region was mutated to check for differences in translation of the TNF- α gene during serum-positive conditions and serum-starved conditions. Additionally, there is a 20 nucleotide upstream region to which miR-130a is known to bind that was mutated. The following sequence of deoxyribonucleotides comprise that upstream region

5' GTGTTCACGTTTGTATTTAT 3'

The seven nucleotides that are underlined are known as the seed region of miR-130a as described by previous experiments done in the Vasudevan lab.

Members of the Vasudevan lab created four plasmids:

- 1) the ARE region and seed region were kept wild-type
- 2) the ARE region was mutated and the seed region was kept wild-type
- 3) the ARE region was mutated and the seed region was mutated
- 4) the ARE region was kept wild-type and the seed region was mutated

METHODS

1. Preparation of Media for THP1 and HEK293 Cells

All media and serum were stored at 4°C when not in use. Media for both cell types were prepared under the cell culture hood. RPMI media by Sigma-Aldrich was prepared for THP1 cells and DMEM media by Sigma-Aldrich was prepared for HEK293 cells. In order to thaw the serum, the container was kept in the 37°C incubator and then heat inactivated at 56°C for 30 minutes in a water bath. 50 milliliters (mL) of serum was added to RPMI media and 50 mL of serum was added to DMEM media for all preparations. During daily use, DMEM and RPMI media was stored in the 37°C incubator. Finally, antibiotics and nutrients were added to each container of media. Antibiotics were stored at 4°C and moved to the 37°C incubator the morning of preparation. 5 mL of Penicillin and streptomycin (P/S) and 5 mL of L-Glutamine (LGlu) were added to each container. Each container was initialed for identification purposes.

2. Splitting HEK293 Cells

In preparation for the splitting of HEK293 cells, 10% Fetal Bovine Serum (FBS) and 1x Dulbecco's Phosphate-Buffered Saline (DPBS) was placed in the 37°C incubator for one hour. Then, plates were removed from the incubator and checked under the microscope for confluency. 10 mL of the media was removed and placed into the waste container. The cells were washed one time with 7 mL of DPBS added to the side of the plate. The plate was then swirled to wash, and the DPBS was removed slowly by tilting the plate to the side. Then, 2 mL of DPBS was added to each plate from the top. Additionally, 200 microliters (uL) of 10x trypsin was added drop-by-drop all over the

plate from the top in order to free adherent cells. The cells were then incubated for three to five minutes and then checked under the microscope to confirm loss of adherency. It is important that the cells are not trypsinized for more than this time as clumps may start to form. Three mL of DMEM was then added to each plate to inactivate the trypsin and mixed by tilting the plate and pipetting the DMEM repeatedly with force. All cells were then placed in a 15 mL centrifuge tube and placed in a centrifuge and spun at 1,000 to 2,000 revolutions per minute (rpm) for 5 minutes at room temperature. The supernatant was removed and 4 mL of fresh DMEM was added to each 15 mL tube that contained the pellet of cells. The cells were pipetted up and down to re-suspend gently so that they would not cluster together. Then, 8 mL of DMEM and 1 mL of FBS was added to each fresh plate. 1 mL of the cell suspension was added to each new plate. The plate was checked under the microscope to make sure they properly adhered and then placed in the 37°C incubator.

3. Counting HEK293 Cells

In order to count, the HEK293 cells were removed from the plate, so all steps outlined for splitting HEK293 cells were repeated. At the re-suspension step after centrifugation, 10 uL of mixture was removed and placed in a hemocytometer at an angle. All cells were counted in the four quadrants as outlined in the diagram below (Figure 1).

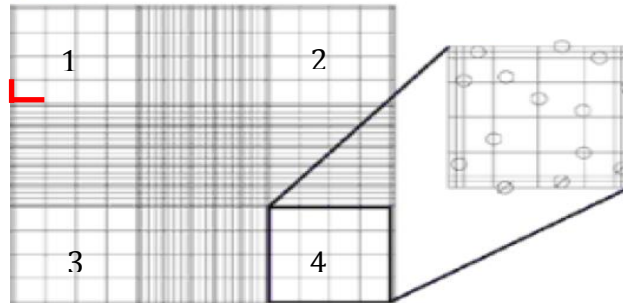


Figure 1. Diagrammatic representation of cell counting using a hemocytometer.

The number of cells counted in all four quadrants was then divided by 4 to get the average number of cells in 0.1 uL of media. That number was then multiplied by 10,000 to get the number of cells in 1 mL of media. After counting the cells, the hemocytometer was cleaned with ethanol and placed back in storage.

4. Splitting THP1 cells

The ideal confluency of THP1 cells is 0.4-0.8 million cells/1 mL. The cells were checked under the microscope to confirm their morphology. The container was then swirled around to make sure the cells were not adhering to the container. About 15 mL of cells were removed with a pipette and placed into fresh flat containers. Then, 15 mL of RPMI media was placed in each container, labeled, and stacked in the 37°C incubator.

5. Transfecting HEK293 Cells for Experiments

Approximately 18 to 24 hours before transfection, cells were plated in a 2.5 mL complete growth medium in a 6-well plate. Ideally, cells should be 50 to 70% confluent prior to transfection. All cells were removed from a 10 cm plate and placed in a 15 mL tube from 6-well plates. The cells were then centrifuged for 5 minutes at 1500 rpm. While waiting, two 6-well plates were labeled. 3 mL of G0 media was added to each well

of a 6-well plate and 3 mL of serum plus media was added to each well of another 6-well plate. The cells were plated at a density of 2.6×10^5 cells/well. The cells were then incubated overnight. After 24 hours, TransIT-293 Reagent: DNA complex was prepared for the TNF- α experiments. If an eIF5B experiment was being performed, the same method was used. Additionally, Lipofectamine was used as another stressor for the eIF5B experiments. The reagent was brought to room temperature and then vortexed gently before using. 250 μ L of Opti-MEM I Reduced-Serum medium was then placed in a sterile Eppendorf tube. Then, 2.5 μ g of four separate plasmid DNAs were added to each designated Eppendorf tube. The plasmid DNAs were wild-type TNF-alpha ARE (AU-Rich Element) (WT), a mutated 13 position in the micro-RNA seed region of interest with a WT ARE (Mt13WTARE), both positions doubly mutated (Mt13MtARE), and a wild-type 13 position with a mutated ARE (Wt13MtARE). Finally, 25 ng of Renilla plasmid was added to each sample in order to control for transfection efficiency after harvesting the cells. The mixtures were pipetted gently. Then 7.5 μ L of TransIT-293 Reagent was added to the diluted DNA mixture and pipetted again. The tubes were left to incubate at room temperature for 15 to 30 minutes. The TransIt-293 Reagent: DNA complexes that were prepared were then added to each designated well for the specific plasmids in a 6-well plate. The culture vessel was gently rocked back and forth and from side-to-side to evenly distribute the TransIT-293 Reagent: DNA complexes. The cells were then incubated for 24 to 72 hours. The cells were then harvested and assayed. Below is a replica of the two plates in which the transfections took place (Figure 2).

Serum-Positive plate (6-well) – 300,000 HEK293 cells

| | | |
|--|---|---|
| WT (25 ug) + Renilla (25 ng) + 7.5 ul TransIT Reagent + HEK293 | Mt13WTARE (25 ug) + Renilla (25 ng) +7.5 ul TransIT Reagent + HEK293 | Mt13MtARE (25 ug) + Renilla (25 ng) + 7.5 ul TransIT Reagent + HEK293 |
| Wt13MtARE (25 ug) + Renilla (25 ng) + 7.5 ul TransIT Reagent + HEK293 | Serum (No plasmid) | Serum (No plasmid) |

G0 Media plate (6-well) – 200,000 HEK293 cells

| | | |
|--|---|---|
| WT (25 ug) + Renilla (25 ng) + 7.5 ul TransIT Reagent + HEK293 | Mt13WTARE (25 ug) + Renilla (25 ng) +7.5 ul TransIT Reagent + HEK293 | Mt13MtARE (25 ug) + Renilla (25 ng) + 7.5 ul TransIT Reagent + HEK293 |
| Wt13MtARE (25 ug) + Renilla (25 ng) + 7.5 ul TransIT Reagent + HEK293 | Serum (No plasmid) | Serum (No plasmid) |

Figure 2. S+ and G0 replica plates for transfecting plasmids into HEK293 cells.

6. Harvesting HEK293 Cells

When HEK293 cells were ready for harvesting, the G0 media and serum-positive media was removed from each 6-well plate. Each well was then washed with 3 mL of PBS and then poured into the waste container again. 5 mL of PBS was then added and then the plate was tilted and scraped. The cells were then pipetted and transferred to a small Eppendorf tube. Each separate well had an Eppendorf equivalent to which the cells were transferred. Each well was then washed with 2 mL of PBS to ensure that all cells were collected. The 6-well plates were discarded and the Eppendorf tubes were centrifuged at 4°C for 5 minutes. The supernatant was then discarded and the sample was flash frozen on dry ice and then stored in the -80°C freezer in 1x passive lysis buffer (PLB). Then a dual luciferase assay was conducted to check for luciferase levels in the cells of interest in both serum-positive and G0 conditions.

7. Dual Luciferase Assay

In order to measure translation levels in the cells of interest, the dual luciferase assay was performed. Cells were removed from the -80°C freezer and kept at room temperature for five minutes to thaw. Once thawed, 10 uL of cell lysate was removed and placed in a new Eppendorf tube. The lysate was pipetted up and down to evenly distribute the contents. Then, 16 uL of Stop and Glo substrate was added to 800 uL of Stop and Glo buffer (50x) for preparation of the . The Promega protocol was run. After adding 100 uL of luciferase reagent (LAR III) to the sample, the Eppendorf tube was placed in a luminometer, the lid was closed, and luminescence was measured. Then, 100 uL of the

Stop and Glo mix was added, which stops the luciferase activity and causes the cells to glow.

8. RNA Isolation from HEK293 cells

About 150 uL of 5 mg/ml Proteinase-K buffer, which has 10% Sodium Dodecyl Sulfate, was added to the cell lysate. The Proteinase-K buffer helps digest proteins and the 10% SDS helps breakdown the cell membrane. The pellet was then disturbed by pipetting in order to homogenize the sample. It is important to remember to change the tip so that the G0 and serum-positive cells do not mix. The Eppendorf tubes with the samples were then vortexed in order to completely break the membrane and digest the protein. Then, the tubes were set at room temperature for 10 minutes. 1 mL of Trizol was then added to each tube. Trizol has guanidinium thiocyanate and is used for RNA extraction. It is light sensitive and often stored in a dark-colored glass container. The tubes were then vortexed and incubated at room temperature for five minutes. Then, 200 uL of chloroform was added and the sample was vortexed again. The samples were then centrifuged at 12,000g for 15 minutes. While waiting for centrifugation, new 1.5 mL Eppendorf tubes were prepared. To each tube, 1 uL of glycogen, 30 uL of sodium acetate (3M, pH=5.2), and 500 uL of 2-isopropanol was added. The centrifuged sample was immediately placed on ice. The aqueous layer was removed from the samples and added to the new 1.5 mL Eppendorf tubes that were prepared. The aqueous layer contains RNA and the chloroform and trizol are in the organic layer. The samples were then inverted up and down gently 3 to 5 times, creating ripples, which signals that RNA is present. The samples were then placed in the -80°C freezer for precipitation. The organic layer that contains the

chloroform and trizol was then discarded into the appropriate waste container. After 24 hours, the samples were removed from the freezer and centrifuged at 20,000g for 40 minutes. The pellet RNA was identified and the supernatant was removed. 1 mL of 70% ethanol was added to each tube to wash the RNA and then removed. Then, a quick spin was performed for a couple seconds to remove the alcohol at 1000 rpm at 4°C. Then, a p10 pipette was used to remove the rest of the ethanol. The sample was then dried on ice with an open lid for five minutes.

9. RNA Clean-Up

About 350 uL of Buffer RLT from Qiagen RNeasy® Mini kit was added to the sample and mixed using a pipette. Then, 250 uL of ethanol was added to the dilute RNA and mixed using a pipette. Then, 700 uL of the sample was transferred to an RNA easy mini spin column, which was placed in a 2 mL collection tube. The lid was closed gently and the sample was centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded as waste. Then, 500 uL of buffer RPE from Qiagen RNeasy® Mini kit was added to the RNA spin-easy column, the sample was spun for 15 seconds at 10,000 rpm and the flow-through was discarded again. The RNA easy-spin column was then placed in a 1.5 mL collection tube and labeled. Approximately 30 to 50 uL of RNAase free water was added directly to the spin column membrane, the lid was closed gently, and the sample was centrifuged for 1 minute at 8,000g or 10,000 rpm to elute the RNA.

10. Measuring RNA concentration

The nanodrop was used to measure the amount of RNA isolated. 1 uL of RNAase free water was first loaded onto the nanodrop to bring the machine to baseline. Then the

surface of the nanodrop was cleaned with a KimWipe, and 1 uL of the sample of interest was added to the nanodrop and the RNA concentration was measured and recorded.

11. cDNA preparation

Two mixes were prepared. The first was an RNA mix and the second was a cDNA synthesis mix. In the RNA mix, 5 uL of RNA, 1 uL of Random Hexamer, 2 uL of dNTP, and 2 uL of water were added. In the cDNA synthesis mix, 4 uL of 5x RT Buffer, 2 uL of 50 mM MgCl₂, 2.0 uL of 0.1M DTT, 0.5 uL of RNase inhibitor, 0.5 uL of Superscript III (RNase transcriptase enzyme), and 1.0 uL of water were added. After making the RNA mix, it was mixed gently and placed in 85°C for 5 minutes to disrupt the secondary structure and allow all random hexamers to bind. Then, the samples were placed on ice for 1 minute, which anneals the RNA to the hexamers. The samples were centrifuged for a couple seconds and then the 10 uL of cDNA synthesis mix was added. Then, the samples were placed in the PCR machine with the following protocol: 25°C for 10 minutes, 50°C for 50 minutes, 85°C for 5 minutes. Then, 1 uL of RNase H was added, which degrades the RNA. The samples were placed in the 37°C incubator for 20 minutes and then the samples were stored in the -20°C freezer.

12. Quantitative-PCR (q-PCR)

The cDNA that was prepared was diluted 1:10. Then, the primer master mix was prepared, where each primer was 50 ng/uL. Then, 3.5 uL of primer mix and 17.5 uL of SYBR green were added to each tube. The tubes were then vortexed and spun down for 5 to 10 seconds at 1,000 rpm. Then, 10 uL triplicates were pipetted in a 384-well plate, making sure no bubbles were formed. The samples were then run on an existing template

program. The primers that were used for the four plasmids of interest were FF3END3 and FF10.5, the primers used for Renilla were Ren1 and Ren2, and the primers used for tRNA-lysine were tRNA-Lys-5 and tRNA-Lys-3. The following sequences are outlined for each primer. Primers to tRNA-Lys were used as follows: tRNA-Lys-5: GCCCGGATAGCTCAGTCGGTAGAG and tRNA-Lys-3: CGCCCGAACAGGGACTTGAACCC. Primers to as an alternative control were Ren1: CCATGATAATGTTGGACGAC and Ren2: GGCACCTTCAACAATAGCATTG. Primers to Firefly, which was exogenously introduced were FF-F3: TTCCATCTTCCAGGGATACG and FF-R3: ATCCAGATCCACAACCTTCG.

Below is replica of the qPCR plate that was run in order to measure translation efficiency (Figure 3). Even though the plate was a 384-well plate, only the wells that were used for the experiment are being outlined for display purposes. All calculations to measure luciferase activity and translation efficiency were done using Microsoft Excel.

| qPCR plate to measure translation efficiency | | | |
|--|------------------|------------------|------------------|
| Mt13WTARE S+ FF | Mt13WTARE S+ FF | Mt13WTARE S+ FF | Mt13WTARE S+ FF |
| Mt13MtARE S+ FF | Mt13MtARE S+ FF | Mt13MtARE S+ FF | Mt13MtARE S+ FF |
| Wt13MtARE S+ FF | Wt13MtARE S+ FF | Wt13MtARE S+ FF | Wt13MtARE S+ FF |
| WT S+ FF | WT S+ FF | WT S+ FF | WT S+ FF |
| Mt13WTARE G0 FF | Mt13WTARE G0 FF | Mt13WTARE G0 FF | Mt13WTARE G0 FF |
| Mt13MtARE G0 FF | Mt13MtARE G0 FF | Mt13MtARE G0 FF | Mt13MtARE G0 FF |
| Wt13MtARE G0 FF | Wt13MtARE G0 FF | Wt13MtARE G0 FF | Wt13MtARE G0 FF |
| WT G0 FF | WT G0 FF | WT G0 FF | WT G0 FF |
| Mt13WTARE S+ | Mt13WTARE S+ | Mt13WTARE S+ | Mt13WTARE S+ |
| Mt13MtARE S+ | Mt13MtARE S+ | Mt13MtARE S+ | Mt13MtARE S+ |
| Wt13MtARE S+ | Wt13MtARE S+ | Wt13MtARE S+ | Wt13MtARE S+ |
| WT S+ | WT S+ | WT S+ | WT S+ |
| Mt13WTARE G0 | Mt13WTARE G0 | Mt13WTARE G0 | Mt13WTARE G0 |
| Mt13MtARE G0 | Mt13MtARE G0 | Mt13MtARE G0 | Mt13MtARE G0 |
| Wt13MtARE G0 | Wt13MtARE G0 | Wt13MtARE G0 | Wt13MtARE G0 |
| WT G0 | WT G0 | WT G0 | WT G0 |
| Mt13WTARE S+ Lys | Mt13WTARE S+ Lys | Mt13WTARE S+ Lys | Mt13WTARE S+ Lys |
| Mt13MtARE S+ Lys | Mt13MtARE S+ Lys | Mt13MtARE S+ Lys | Mt13MtARE S+ Lys |
| Wt13MtARE S+ Lys | Wt13MtARE S+ Lys | Wt13MtARE S+ Lys | Wt13MtARE S+ Lys |
| WT S+ Lys | WT S+ Lys | WT S+ Lys | WT S+ Lys |
| Mt13WTARE G0 Lys | Mt13WTARE G0 Lys | Mt13WTARE G0 Lys | Mt13WTARE G0 Lys |
| Mt13MtARE G0 Lys | Mt13MtARE G0 Lys | Mt13MtARE G0 Lys | Mt13MtARE G0 Lys |
| Wt13MtARE G0 Lys | Wt13MtARE G0 Lys | Wt13MtARE G0 Lys | Wt13MtARE G0 Lys |
| WT G0 Lys | WT G0 Lys | WT G0 Lys | WT G0 Lys |

Figure 3. Replica of qPCR plate used to measure translation efficiency.

The above plate was color-coded to outline that the first set was used to measure firefly's translation efficiency. The second set was used to measure Renilla's translation efficiency. The third set is used to measure tRNA-Lysine's translation efficiency. Firefly and Renilla were used to calculate translation efficiency and tRNA-lysine was used as a control. Each primer master mix was replicated three times and then averaged to calculate translation efficiency after the qPCR was performed.

The formula that was used to calculate translation efficiency is as follows:

$$\frac{[\text{Firefly Luciferase levels} / \text{Renilla Luciferase levels}]}{[\text{Firefly RNA levels} / \text{Renilla RNA levels}]}$$

RESULTS

The results indicated in this section provide insight as to what degree the TNF- α transcript was translated by measuring luciferase activity and translation efficiency in both serum-positive conditions and serum-starved G0 conditions. The results also show the levels of two proteins, eIF5B and FXR1 during both serum-positive and serum-starved G0 conditions. The results were then analyzed to deduce a possible explanation as to how miRNA and ARE interplay result in conditions that are suitable for cancer cells.

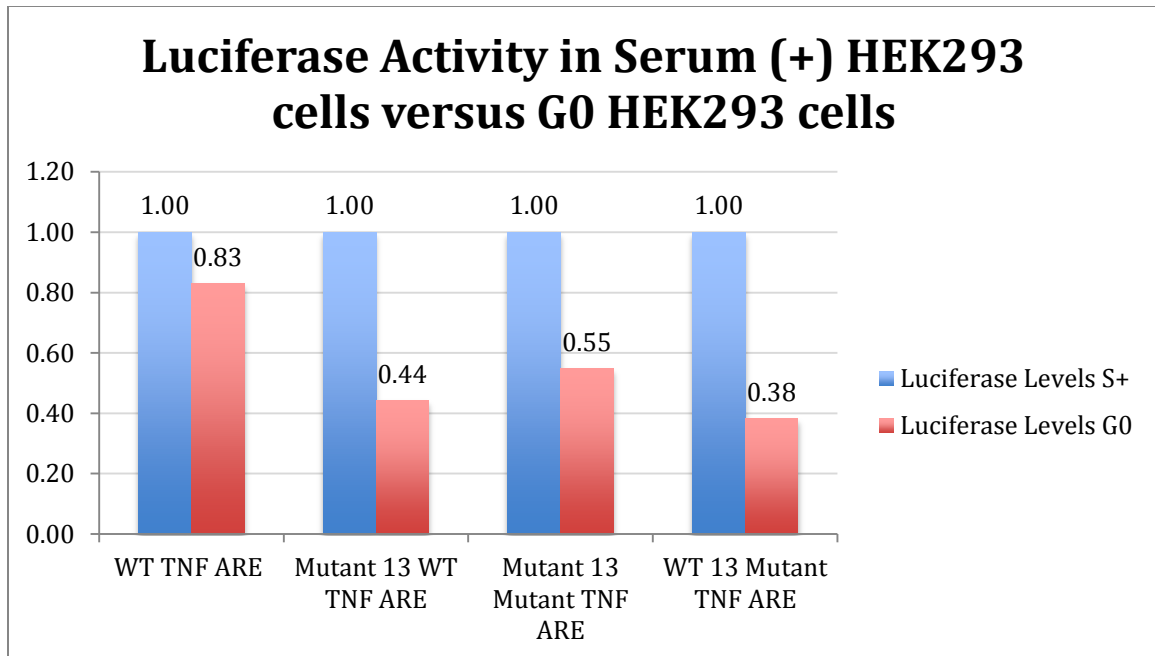


Figure 4. Luciferase Activity in Serum+ HEK293 cells in comparison to G0 HEK293 cells. Results are averaged over 5 experimental replicates. All S+ levels were standardized to 1.00 and compared to G0 HEK293 cells to understand the effect of mutating the ARE region of TNF- α or the seed region where miR-130a binds, mutating both, or keeping both regions wild-type.

As seen above, when the upstream seed region of miR-130a was mutated with the TNF- α ARE remaining wild-type, the luciferase activity decreases in the G0 cells as compared to the S+ cells, which indicated a decrease in expression of the ARE in the G0 cells, which may be a result of less miR-130a being able to bind to the seed region. When the seed region and the ARE region were both mutated, there still appears to be a decrease in expression in the G0 cells as compared to the S+ cells, but not as sharp of a decrease as compared to when only the seed region is mutated. When only the ARE was mutated, and the seed region remains wild-type, expression levels decrease again. The graph above was averaged over five experimental replicates. No firm conclusions can be made as a control was not used. However, according to the above results, it is evident that when either or both the seed region and the ARE region are mutated, expression levels

decrease as compared to when both regions are wild-type. This shows support for the fact that miR-130a plays an important role in the regulation and expression of TNF- α at some level or another.

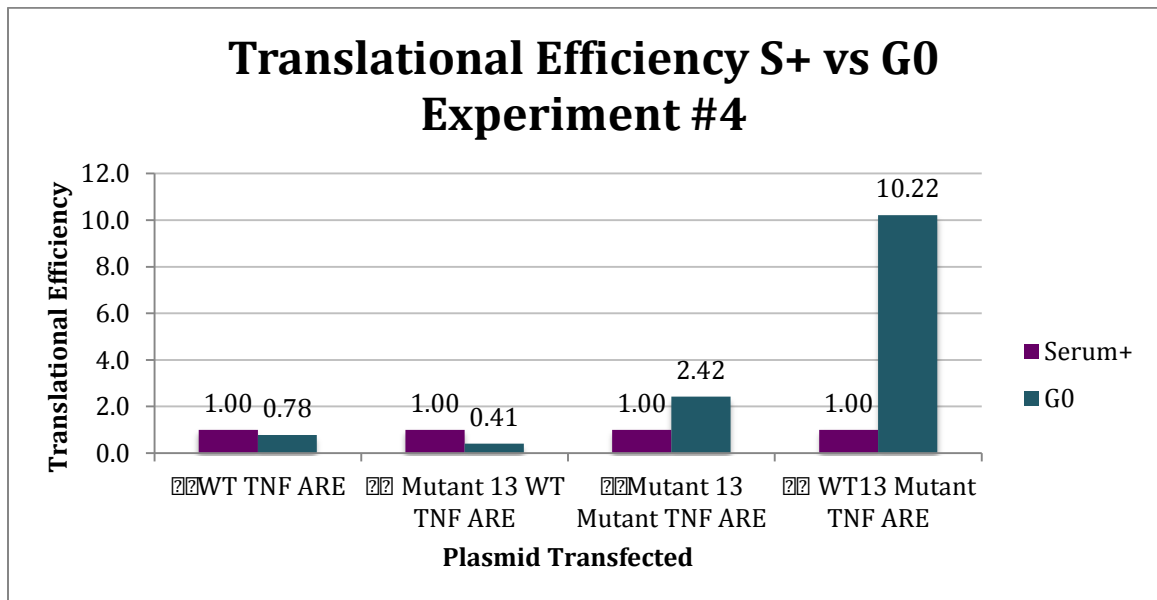


Figure 5. Trial 1-Translational Efficiency in HEK293 Cells Serum+ versus G0 conditions. All S+ levels were standardized to 1.00 and compared to G0 HEK293 cells to understand the effect of mutating the ARE region of TNF- α or the seed region where miR-130a binds, mutating both, or keeping both regions wild-type.

In the first three experiments that were conducted to isolate RNA, prepare cDNA, and then perform qPCR in order to assess translational efficiency, there were loading problems into the 384-well plate and therefore, translational efficiency was not assessed from those experiments. In experiment 4, which is noted in Figure 5, the translational efficiency in the cells under G0 conditions was slightly lower than the cells in serum-positive conditions when both regions were kept wild-type. When the seed region where miR-130a is known to bind is mutated, the translational efficiency decreases by more than half. Surprisingly, when the seed region and the ARE region are both mutated, the

translational efficiency increases over twofold. When only the ARE region is mutated and the seed region is kept wild-type, the translational efficiency increases over tenfold. All serum-positive conditions were standardized to 1.00 and G0 conditions were compared in relation to the serum-positive conditions.

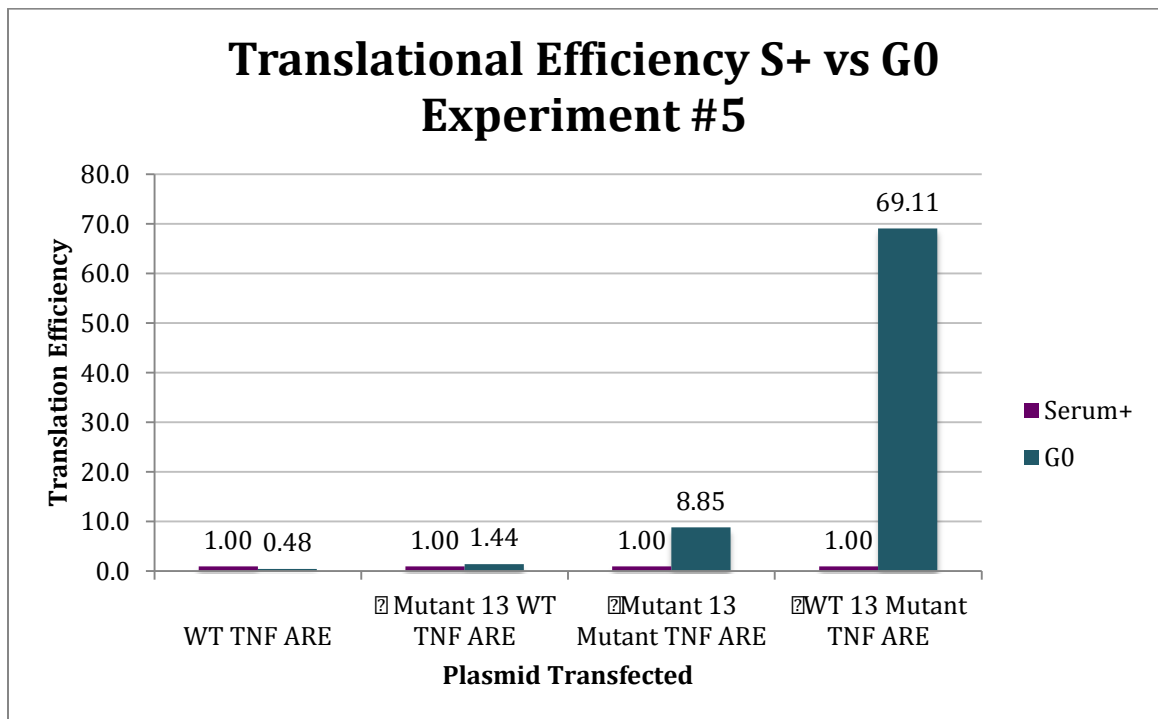


Figure 6. Trial 2-Translational Efficiency in HEK293 Cells Serum (+) versus G0 conditions. All S+ levels were standardized to 1.00 and compared to G0 HEK293 cells to understand the effect of mutating the ARE region of TNF- α or the seed region where mir-130a binds, mutating both, or keeping both regions wild-type.

In Experiment 5 shown in Figure 6, when both regions were kept wild-type, the translational efficiency decreased by about half. When only the seed region was mutated, the efficiency increased slightly, but less than double. When both regions were mutated as in Experiment 4, the translational efficiency increased by eightfold. When only the ARE region was mutated, the translational efficiency increased by almost 70 fold. In this bar graph, a difference in the direction of the second plasmid, Mutant 13 WT TNF ARE,

was seen. In Experiment 4, the translation efficiency decreased as compared to serum-positive conditions. In Experiment 5, the translation efficiency increased as compared to serum-positive conditions.

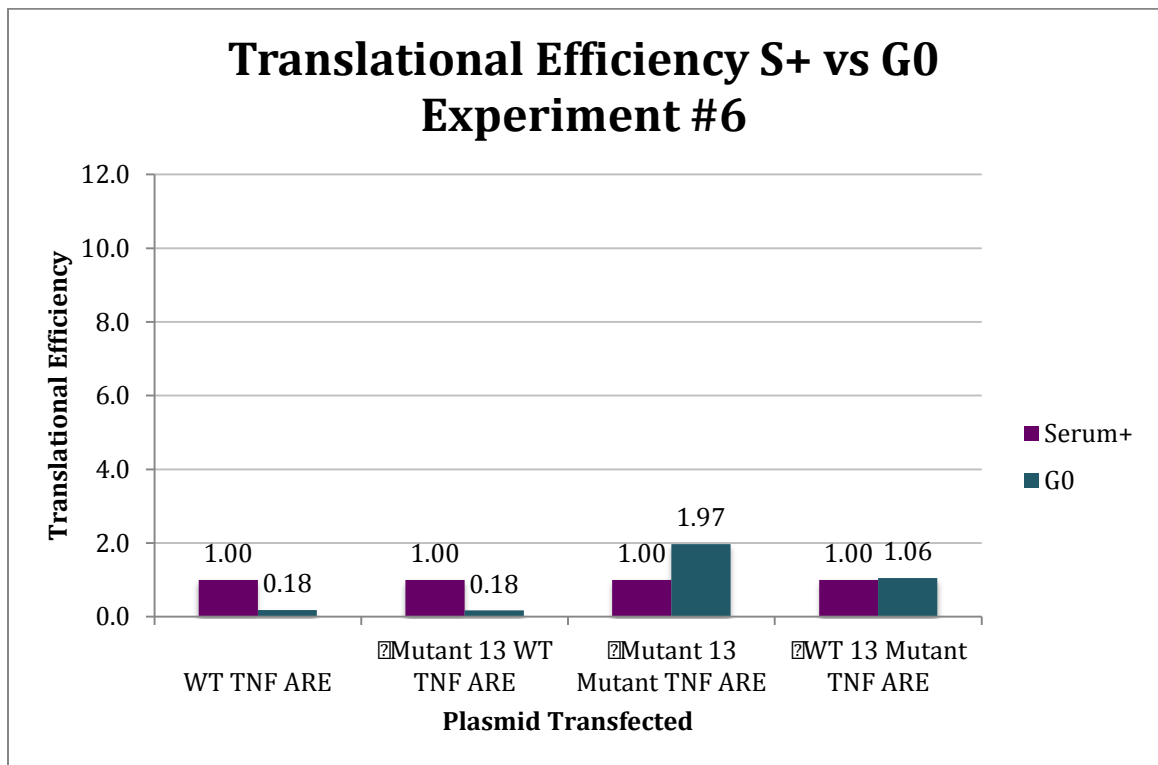


Figure 7. Trial 3-Translational Efficiency in HEK293 Cells Serum+ versus G0 conditions. All S+ levels were standardized to 1.00 and compared to G0 HEK293 cells to understand the effect of mutating the ARE region of TNF- α or the seed region where miR-130a binds, mutating both, or keeping both regions wild-type.

In Experiment 6, when both regions were kept wild-type, the translational efficiency decreased by more than fifty percent. When the seed region was mutated, the translational efficiency decreased to the same amount when kept wild-type. When both regions were mutated, the efficiency increased almost twofold. Lastly, when only the ARE region was mutated, the translational efficiency remained approximately the same.

There appears to be no consistency in the three experiments as to whether the translational efficiency was increased or decreased for the TNF- α gene when the different regions were mutated in the serum-positive state or the G0 state. No firm conclusions can be made according to this data. Two out of the three experiments do show an increase in efficiency when the ARE region is mutated. All experiments show an increase in efficiency when both the ARE and the seed region are mutated.

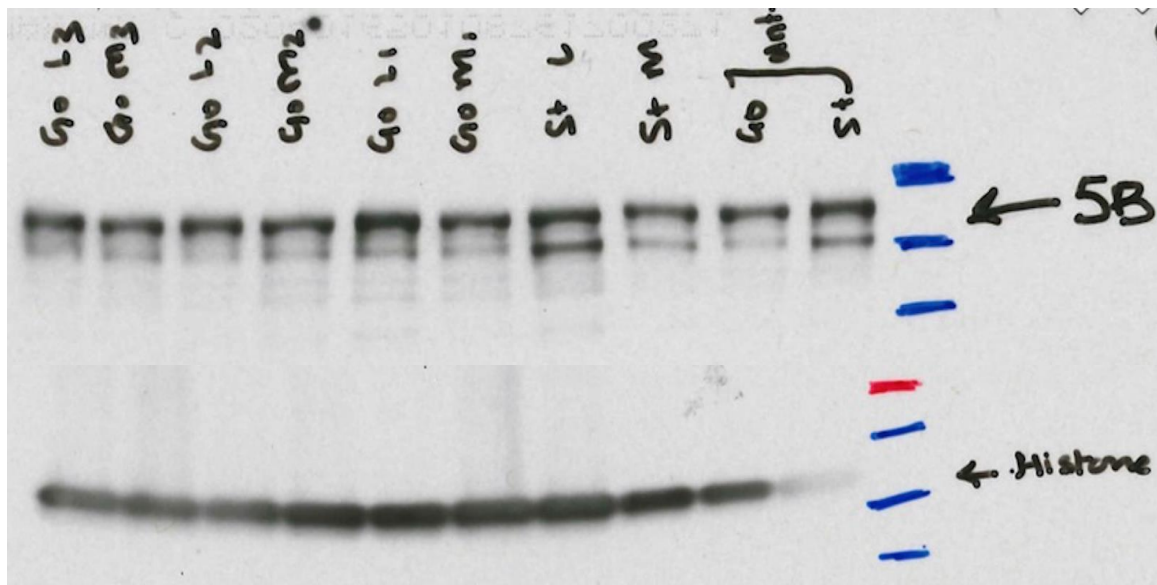


Figure 8. Western Blot assessing levels of eIF5B Protein and Histone Levels (as Control) 24 hours after transfection with lipofectamine and 24 hours after transfection with Mirus. In comparison to untransfected G0 cells, 5B levels increase when transfected with lipofectamine. In comparison to untransfected S+ cells, 5B levels show an increase when transfected with lipofectamine. Transfected S+ cells show higher levels of eIF5B in comparison to transfected G0 cells.

In the western blot (Figure 8), cells that were grown in serum positive conditions and G0 conditions were assessed for protein levels of eIF5B. Histone was used as a control for the eIF5B protein. In the above figure, eIF5B levels increase when transfected with lipofectamine, a stressor to the cells, in G0 conditions in comparison to untransfected cells.

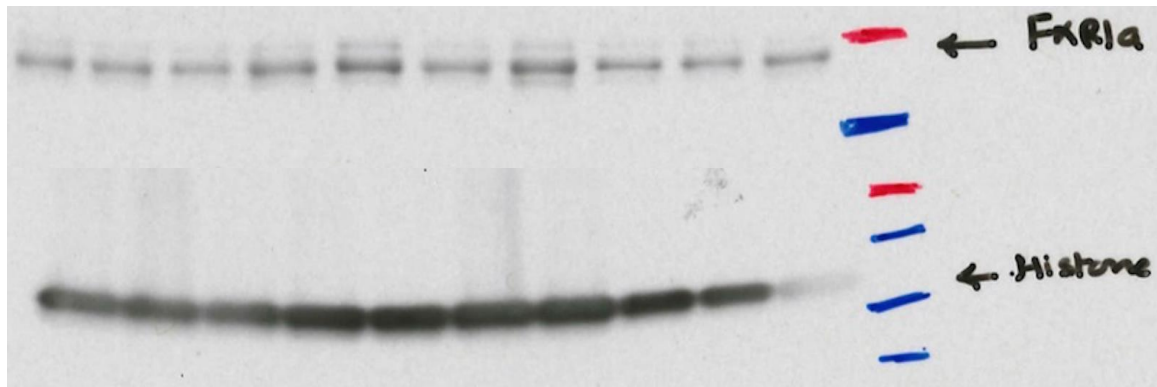


Figure 9: Western Blot assessing levels of FXR1 Protein and Histone Levels (as Control) 24 hours after transfection with lipofectamine and 24 hours after transfection with Mirus. FXR1a levels increase 24 hours after transfection in S+ cells transfected with lipofectamine, but decrease in levels after transfection in G0 cells.

In the western blot shown in Figure 9, cells that were grown in serum-positive conditions and G0 conditions were assessed for protein levels of FXR1, a protein that is known to bind to AGO2 during G0 conditions. Histone was used as a control for the protein. In the above figure, FXR1 levels increase when transfected with lipofectamine in serum-positive conditions. However, they decrease in levels when transfected with lipofectamine or Mirus in G0 conditions 1 day after transfection. This indicates that there may be a connection between eIF5B and FXR1 protein levels in the G0 state. As eIF5B increases, there appears to be a decrease in FXR1 levels. There may be an antagonistic correlation between the two and further experiments may elucidate more information regarding this correlation.

DISCUSSION

Quiescence is an interesting cell-cycle stage in that many changes may occur, some of which are still being discovered. General translation exhibits a reduction in the quiescent stage, yet specific genes may be turned on in order for the cell to stay viable.²⁵ It was shown by Vasudevan et al. that eIF5B acts as an inhibitor to G0-like stages by

preventing arrest during the cell-cycle to G0 transition if serum-starved for 1 day.²⁵ Experiments done by Vasudevan et al. were in THP1 cells. This study aimed to see if other cell lines exhibited the same behavior. HEK293 cells were used in these experiments. In the HEK293 cells, eIF5B also increased in transfected G0 (stress-induced) cells in comparison to untransfected G0 cells, which may be indicative that eIF5B plays a larger role in translating certain proteins in the stress-induced state, as opposed to when the cells are in the G0 state, without a stress-inducer, such as lipofectamine or Mirus. This is consistent with one of eIF5B's roles in taking part in translating specific mRNAs that are needed in stress-associated states. An interesting experiment to conduct would be to check whether prolonged G0 states have a different effect on eIF5B levels in HEK293 cells. Another experiment that could provide insight to replicate work in THP1 cells by Vasudevan et al. would be to overexpress eIF5B in cells and check whether cells entered the G0 phase or experienced cell death. In their study, they found that when eIF5B was overexpressed, more cells, on average, experienced cell death.

The FXR1 protein was also studied in this analysis. Vasudevan et al. showed that in G0 cells, the interaction between proteins GW182 and AGO2 is reduced.¹⁶ Instead AGO2 binds to FXR1 and creates a complex in G0 cells, which activates translation. In 1 day serum starvation in HEK293 cells, however, FXR1 levels show a decrease, thereby indicating that eIF5B may cause FXR1 to decrease 1 day after serum starvation. To some degree, it can be concluded that FXR1 is decreased 1 day after serum starvation, when eIF5B is upregulated and is an antagonist of G0, but once prolonged serum starvation

occurs, FXR1 protein levels rise, and creates a complex with AGO2, which activates translation specific to G0 cells. This shows that there is some complementarity between the two proteins during translation and further experiments need to be conducted to confirm a link between the two. Although prolonged serum starvation shows different results, gaining insight on one day serum starvation is helpful as it may provide information on delaying the G0 state.

Since a possible indication occurs that there is a relationship between FXR1 and eIF5B proteins during translation, it is important to further develop experiments to assess if they come back to normal levels in both serum-positive conditions and serum-starved G0 conditions. A possible second project to assess the importance of both eIF5B and FXR1 during translation would be to check whether FXR1 levels went back up and eIF5B levels went back down to normal basal levels. Since FXR1 was shown to be important by binding to AGO2 in the quiescent state in order for activation of translation to occur, it would be expected that FXR1 goes back up to normal levels so that certain proteins needed for the cell to remain viable can be translated.

In this analysis, the TNF- α gene and its translation efficiency was also studied. The effect of mutating a seed region where miR-130a is known to regulate was analyzed in serum-positive and G0 cells. Moreover, the effect of mutating the ARE region of TNF- α was analyzed in serum positive and G0 cells. The TNF- α gene plays an important role in the inflammatory process involved in cancer, and may provide insight as to how miRNAs contribute to the regulation of its translation during the G0 state. Unfortunately,

the experiments did not provide any conclusive results, yet further hypotheses and tests can be conducted to elucidate the role of miR-130a to the expression of the TNF- α gene. However, there may be an explanation to the variability in the experiments conducted in HEK293 cells to assess TNF- α expression. This, in part, may be due to the fact that the plasmids were transfected instead of nucleofacted, wherein the plasmids are inserted directly into the nucleus instead of the cytoplasm. In a study done by Vasudevan et al.,²⁷ their results suggested that AGO2, the protein that binds to GW182 in serum conditions and FXR1 in G0 conditions to promote translation, is present in the cytoplasm more so than in the nucleus of mammalian cells. When cytoplasmic AGO2 is bound to large complexes, such as GW182 and miRNAs, it appears to be repressive of translation. However, when bound to FXR1 in G0 states, it becomes an activating factor for translation. Their results also provided even more insight. They found that the AGO2-FXR1 complex was not detected in the cytoplasm of low density proliferating cells and was also reduced in the cytoplasm of oocytes. When FXR1 was overexpressed, it led to an increase in binding with AGO2 in the cytoplasm of oocytes and proliferating cells, which took the place of the nuclear AGO2-FXR1 complexes.²⁷

This finding indicates that translation has different fates in the cytoplasm versus in the nucleus. In the experiments conducted in this project looking at TNF- α expression, the plasmids were inserted into the cytoplasm using transfection, and the variability in the translation efficiency may be due to the levels of AGO2 in the cytoplasm. If the plasmids instead are nucleofacted into the nucleus where AGO2 is known to be freer in G0 serum-starved conditions, then possibly the variation would decrease, and better conclusions can

be made based off the resulting findings. It is important that these experiments be re-done by nucleofecting plasmids instead of transfecting them into the cytoplasm.

Theoretically, variability in the TNF- α experiments can also possibly be explained by the decrease in FXR1 levels in one day serum starved cells. Since FXR1 is needed for activation of certain genes, the decrease in FXR1 as eIF5B levels increased can explain why the TNF- α gene had significant variability in its translation in the G0 state. A possible way to check whether this is true would be to overexpress FXR1 and observe whether there was any consistency in the translation efficiency of the TNF- α gene.

Finally, in a study done by Chongtae et al., the researchers examined miR-130 levels in white adipose tissue from high-fat diet mice and adipocytes that were stimulated by TNF- α .²⁶ They found that the miR-130 transcripts were increased after TNF- α stimulation. This showed the researchers that miR-130 levels increased on a transcriptional level during the inflammatory response. They also did chromatin immunoprecipitation assays to show that p65, a transcription factor, binded to the promoter regions of miR-130 after TNF- α treatment. They concluded that TNF- α induced miR-130 to upregulate and cause adipocyte dysfunction.²⁶ This information is important as many cancers are caused by high-fat diet. Further studies and experiments can be designed to show how upregulating p65 or downregulating p65 changes conditions. Moreover, p65 can be blocked from binding to the promoter region of miR-130 to see if a change in adipocyte dysfunction ensues. This would be important to the field of inflammatory responses.

As noted through this project, AREs are important regions of an mRNA's 3'-UTR, a cis-regulatory factor and microRNAs are small noncoding RNA regions that are found upstream of this region, a trans-regulatory factor. MiRNAs play important roles in cytokine regulation, cell proliferation, cell death, and cell transitions. If altered from their normal course, tumor genesis may occur, and downstream, chemo-resistance and metastasis may ensue, causing drastic impediments in treating patients that are suffering from cancer.

Exploring these regulatory mechanisms by noncoding RNAs, microRNAs, AREs, and their interactions with other known proteins such as eIF5B and FXR1 in response to both serum-positive conditions and quiescent serum-starved conditions can explain many aberrations in the cell cycle. Many different cell types should be used as not all cells have the same interactions occurring. Environment, diet, underlying genetic factors, and other contributors may play a huge part in how a cell reacts to given conditions experimentally. Transformations occur on many different levels in the quiescent condition, and allows for many important questions to be raised. Instead of proliferating or dying, these cells remain in an adaptive state that proves to be advantageous to cells in a cancer state and therefore, may cause recurrence several years down the road in a patient even after having successful treatment. It is imperative that these regulatory mechanisms, including miRNA regulation and interactions with known translation regulators, be characterized in a systematic matter. Since it is known that cytokines play a huge role in tumorigenesis and cancer, it is valid that experiments in cytokines would provide insight.

Once these important discoveries are made and contribute to the recent literature, RNA based therapeutics may be developed that can change gene expression of certain quiescent cells for specific cancers. As stated previously, miRNAs have been a recent discovery of gene regulation and expression and their functions continue to be investigated for further knowledge.

LIST OF JOURNAL ABBREVIATIONS

| | |
|----------------------------|---|
| ACS Synth Biol | ACS synthetic biology |
| Annu Rev Nutr | Annual review of nutrition |
| Cell | Cell |
| Cell Cycle | Cell Cycle (Georgetown, Tex.). |
| Cytokine Growth Factor Rev | Cytokine & growth factor reviews. |
| EMBO J | The EMBO Journal |
| FEBS Lett | FEBS Letters |
| Genes and Dev | Genes & development |
| J Interferon Cytokine Res | Journal of interferon and cytokine research: the official journal of the International Society for Interferon and Cytokine Research |
| J Cell Biol | The Journal of cell biology |
| J Cell Sci | Journal of cell science |
| J Mol Biol | Journal of Molecular Biology |
| J Oncol | Journal of oncology |
| J Transl Med | Journal of translational medicine |
| Mol Cell | Molecular Cell |
| Nat Genet | Nature Genetics |
| Nat Rev Mol Cell Biol | Nature reviews. Molecular cell biology. |
| PLoS Biol | PLoS biology |

Proc Natl Acad Sci U S A Proceedings of the National Academy of Sciences of the
United States of America
Science Science (New York, N.Y.)
Sci Rep Scientific reports
Wiley Interdiscip Rev RNA Wiley interdisciplinary reviews. RNA.

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CURRICULUM VITAE

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904-614-5634 | U.S. Citizen | 1989

Professional Profile/Performance Summary

MS/MPH Candidate with experience in academic research and clinical settings. Strategically manage time and handle multiple projects for optimal productivity, improvement, and profitability. Special interest in analyzing outcomes data used for planned decision-making in healthcare and public health sectors. Proficient with Word, Excel, PowerPoint, Outlook, Gantt Charts, R21 and R01 Grant Writing, SAS, R, and Microsoft SQL Software.

Education

- ◆ **Master of Public Health, Epidemiology** **Aug 2012-May 2015**
Master of Science, Medical Sciences *Boston University*
 - Tutor for Introductory Epidemiology, Health Law, Environmental Health, and Health Policy and Management
 - MPH Practicum: Boston University Slone Epidemiology Center
 - ✓ Delivering descriptive tables for analysis of lifestyle factors on fertility outcomes using SAS for nationwide conference
 - ✓ Facilitating and assisting with recruitment and follow-up of participants for Pregnancy Study Online (PRESTO)
 - ✓ Drafted detailed literature review on association between appendicitis and later fertility in women for publication

- ◆ **Bachelor of Science** *Emory University* **Aug 2007-Dec 2010**
Biology; Minor: Religion

Professional Experience

- Boston Children's Hospital ♦ Boston, MA** **Jun 2014-present**
Biostatistician
 - Spearheading NCQA ACO Measures Pilot Project: analyzing and reporting measures determined by NCQA
 - Designing Physician Maps to provide detailed up-to-date provider hierarchy for use in assessing quality and process measures
 - Conducting detailed review and analysis of current Medicaid ACO cost and utilization benchmarks across the nation

- Researching effective solutions in streamlining primary care physician referrals to specialists for performance improvement
- Interacting with clinical staff and biostatisticians to design and generate reports and data for use in pediatric practices
- Extensive use and technical knowledge of claims data, hospital patient data, and ICD-9 diagnostic coding

Massachusetts General Hospital: Cancer Center ♦ Boston, MA Feb 2013- Apr 2015
Graduate Student Research Assistant

- Completed research project on gene expression regulation of cytokine factors in various conditions in cancer models, and assisted post-doctoral fellow with research project aimed at discovering RNA-based therapeutics for cancer patients.
- Analyzed and translated complex data into graphs using Excel; presented findings to lab members/principal investigator.
- Conducted literature reviews in order to present novel ideas and findings in cancer research as part of journal club meetings.

Emory University School of Medicine ♦ Atlanta, GA Oct 2010-May 2012
Lab Manager/ Research Technician

- Maintained and bred transgenic mice colonies, ensured clear communication with vet staff to follow IACUC regulations.
- Managed inventory databases in order to efficiently manage budgetary responsibilities and track shipments of lab supplies.
- Adhered to safety and inspection protocols involving radioactivity and wastes; trained and supervised all personnel.
- Coached and provided mentoring to junior undergraduate students in lab techniques and wrote clear, concise lab protocols.

Roy L. Schneider Hospital ♦ St. Thomas, VI Jun-Aug 2008, 2009
Summer Intern

- Gained valuable patient and physician interaction in histopathology department and Charlotte Kimelman Cancer Institute.
- Attended skin grafting and gall bladder removal surgeries and learned about medical procedures from surgeons.
- Communicated effectively with histopathologist during autopsies by accessioning notes on bodily injuries.
- Measured patient's vital signs and built rapport in an effort to alleviate their frustration during long wait times

Hobbies: avid traveler; photography enthusiast; amateur painter.