Attenuation of Angiomotin Expression via CRISPR in Triple-Negative Mammary Epithelia

A Senior Paper

Presented to

the Department of Biology

of Oral Roberts University

In Partial Fulfillment

of the Requirements for the Degree

Bachelor of Science

by

Riley Andrews

November 2020

Dr. William Ranahan

Table of Contents

List of Tables

List of Figures

Abstract

Breast cancer is the most commonly diagnosed form of cancer among women living in the United States, and it is characterized by uncontrolled cell growth in either the lobules or ducts of the breast tissue. Additionally, breast cancer accounts for the second highest number of cancer deaths, only trailing behind lung cancer. Current efforts to treat this form of cancer include various combinations of chemotherapy, mastectomy, radiation, and endocrine therapy; however, there is still a need for treatments with increased selectivity. Use of the CRISPR/Cas9 gene-editing tool is a method that could be used to directly target cancer cells. This gene-editing tool is guided to the appropriate sequence through a complementary small guide RNA (sgRNA) that can be designed for any gene of interest. Angiogenesis, which is required in order for tumor cells to survive, is the process in which new blood vessels are formed from pre-existing vessels, and angiomotin (Amot) is directly involved in this process. Additionally, various studies have found that Amot enables the proliferation of mammary epithelia through the activation of extracellular signal-regulated kinases (ERK1/2). Therefore, Amot is thought to be a putative oncogene involved in breast cancer. The current study focused on reducing Amot expression in tumorigenic MDA-MB-468 cells via CRISPR. Cancer cells were transfected with varying guide RNA (gRNA) sequences and varying concentrations of CRISPR-containing DNA constructs. qPCR data suggested that the gRNA sequence "Amot 174" was most effective at reducing Amot mRNA transcripts. Amot mRNA reduction correlated with a decrease in proliferating cell nuclear antigen (PCNA) mRNA transcripts, suggesting a decrease in cell viability. Follow up studies will include Western blotting to confirm reduction in Amot protein, cell viability assays to confirm cytotoxicity, and end-point PCR to confirm editing of the Amot gene.

Introduction

A Brief Overview of Cancer

Cancer is a disease that has become the leading cause of death in developed nations. Cancer can impact individuals across all demographics. Each year in the United States, 1 million people are diagnosed with cancer, and 500,000 die from this disease (Klug et al. 2015). The disease of cancer is distinguished by two major characteristics: proliferation and metastasis. Normally, the tendency for cells to proliferate is not a problem; cells need to divide in order to replace dead or damaged cells. However, the proliferation of cancer cells is continuous and does not respond to the normal regulatory signals of the cell cycle (Figure 1). The cell cycle consists

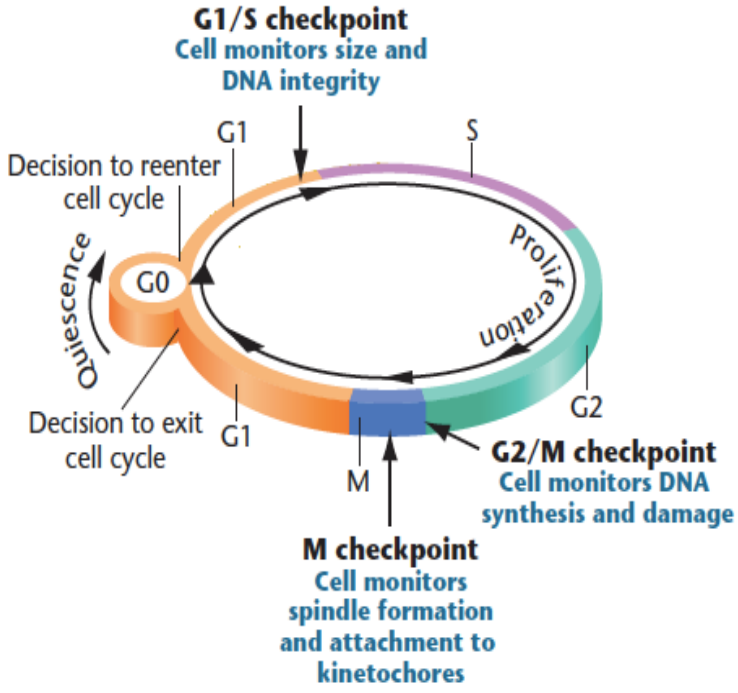

Figure 1: Cell cycle phases and checkpoints (Klug et al. 2015).

of five stages: G1, G0, S, G2, and M. Interphase is the broad term that encompasses the G1, G0, S, and G2 phases, and this is the phase in which the cell spends most of its time. The M phase,

on the other hand, is rapid and is known as mitosis. The G1 phase is characterized by the growth of the cell and preparation for the DNA synthesis stage, called S phase. The G0 phase is a quiescent stage in which the cell remains metabolically active but does not divide; cells enter this phase when they are mature and receive signals to halt growth. In the S phase, the chromosomal DNA is duplicated in order to provide equal genetic material for each daughter cell. During the G2 phase, the cell continues to grow. Finally, in the M phase, the cell divides and produces two identical daughter cells. (Klug et al. 2015). It is whether or not the mature cell is able to remain in the G0 phase at the appropriate time that determines if a cell becomes cancerous. For example, if a tissue does not need to grow, then the cells need to remain in the G0 phase. However, cancer cells do not enter this quiescent phase and continue to proliferate. Proliferation is detrimental to the proper functioning of the organ in which the cancer cells originated. Metastasis, on the other hand, is the tendency for cancer cells to migrate to other organs of the body, and it is deeply problematic for the patient (Klug et al. 2015). The cancerous cells not only move to a new location, but they also spread the cancerous phenotype to the new locations. Cancer can occur in any organ of the body, and it is breast cancer that is of particular interest in the current study.

Breast Cancer Types and Prevalence

Prior to distinguishing the differing types of breast cancer, it is first important to understand the basic anatomy and physiology of the human breast. The breast of the human female is a specialized organ that enables the production of milk during lactation. The breast tissue consists of epithelial lobules, in which the milk is generated, and epithelial ducts that connect the lobules to the nipple (Overview of the Breast - Breast Pathology 2020). The lobules and ducts of the human breast are usually the components of epithelial tissue that are implicated in the development of cancer. The various lobules and ducts (Figure 2) are surrounded by the

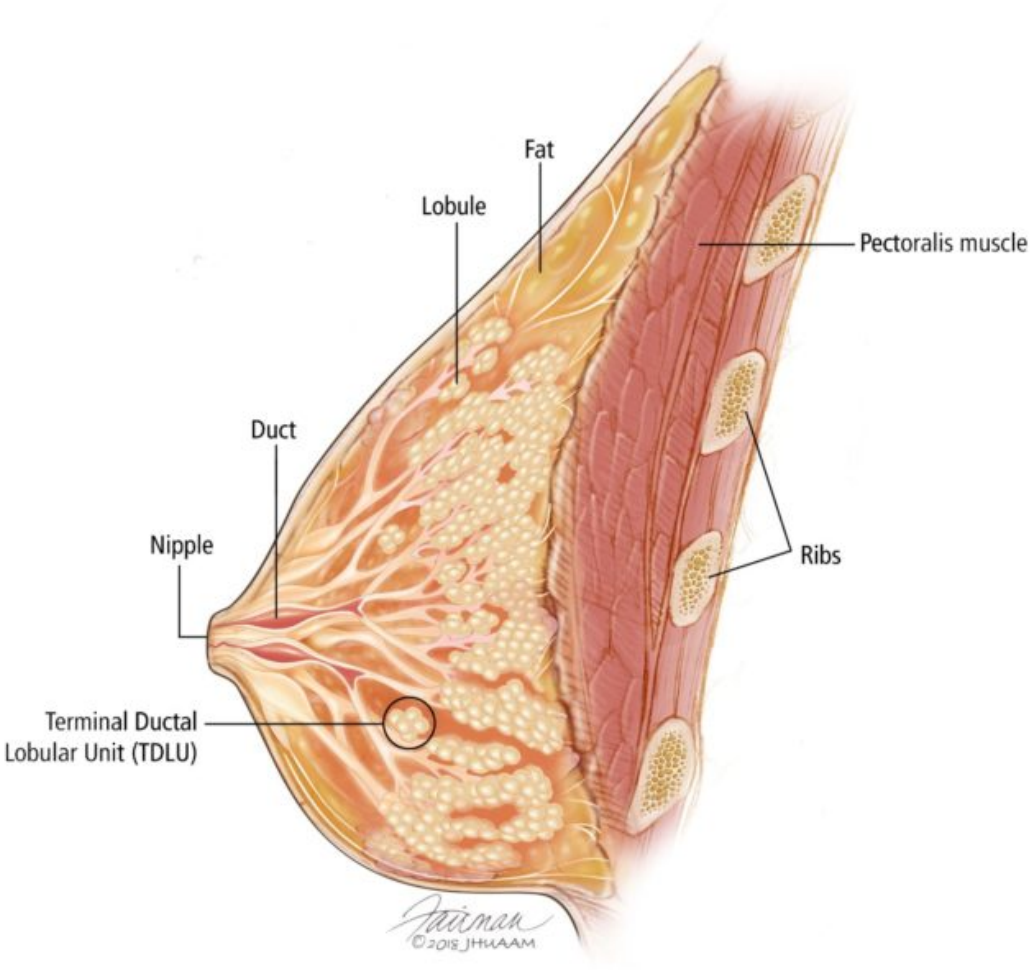

Figure 2: The anatomy of the human breast (Overview of the Breast - Breast Pathology 2020).

fibrous and adipose tissues that make up a large portion of the breast. The breast is positioned on top of the pectoralis muscle of the chest, and breast tissue traverses the distance between the sternum and axilla. The portion of breast tissue that reaches the axilla is known as "the axillary tail of Spence." The structure of the male breast is largely similar to the female breast, except for the fact that the male breast lacks the lobules that are able to produce milk. Blood supply to this organ is provided by the internal mammary artery, and the lymphatic fluid from the breast tissue flows to lymph nodes located in the axilla (Overview of the Breast - Breast Pathology 2020). Given the general anatomy of the human breast, it is much easier to delineate which structures are involved in the differing types of breast cancer. For example, there are two main types of breast cancer: ductal carcinoma and lobular carcinoma. As discussed earlier, the epithelial ducts are those tubes that connect the milk-producing lobules to the nipple. Therefore, ductal carcinoma is characterized by cancer cells that occur in these epithelial ducts. Within the category of ductal carcinoma, there is the classification of invasive ductal carcinoma (IDC) and the classification of ductal carcinoma in situ (DCIS). Ductal carcinoma in situ is a non-invasive type of breast cancer; therefore, this type of breast cancer is not life-threatening. In most cases, the tumor can be surgically removed. However, those who are diagnosed with DCIS have an elevated risk of being diagnosed with IDC later on in life (BreastCancer.org 2018). The category of invasive ductal carcinoma, on the other hand, is potentially life-threatening due to the fact that these cancerous cells are able to invade the surrounding tissues of the breast and the lymph nodes of the axilla. There are many different subtypes of invasive ductal carcinomas, such as tubular carcinoma, medullary carcinoma, mucinous carcinoma, papillary carcinoma, and cribriform carcinoma. Tubular carcinoma is characterized by the formation of small, tubular tumors. Given the slow-growing nature of these tumors, this subtype of IDC is considered to be responsive to

treatment and less aggressive than other subtypes (BreastCancer.org 2018). The medullary subtype forms tumors that are soft and fleshy in appearance; these tumors are also slow-growing and do not usually spread to the lymph nodes. The medullary subtype is relatively rare, and it has been associated with the *BRCA1* mutation (BreastCancer.org 2018). Mucinous carcinoma is distinguished by its tumors that consist of cancer cells that are immersed in a collection of mucin, which is a component of mucus. There seems to be a trend in which diagnoses of mucinous carcinoma are more prevalent in women that have already gone through menopause. It is also important to mention that this subtype is also not very aggressive in nature (BreastCancer.org 2018). The tumors of invasive papillary carcinomas have a definitive border and fingerlike projections, and they are moderate in terms of growth rate of tumors (BreastCancer.org 2018). Lastly, cribriform invasive ductal carcinoma is characterized by tumors in the connective tissues of the breast; these tumors form between the lobules and the ducts, and they are recognizable by "holes" in between the cells of the tumor (BreastCancer.org 2018). The entire category of invasive ductal carcinoma, which consists of all the aforementioned subtypes, is the most common type of breast cancer, making up about 80 percent of all breast cancer diagnoses (BreastCancer.org 2018). The second category of breast cancer, invasive lobular carcinoma, is also the second most common type of breast cancer. In lobular carcinoma, the tumor originates in the lobules of the breast, and the cancerous cells migrate throughout the surrounding breast tissues as well as into the lymph nodes (BreastCancer.org 2018). Given the plethora of subtypes of breast cancer, it is therefore reasonable that breast cancer is the most commonly diagnosed cancer among women in the United States, accounting for about 1 in 3 cancer diagnoses in women. Given the high prevalence in the population, breast cancer is the second highest cause of cancer death, only trailing behind lung cancer (DeSantis et al. 2014). In

the United States, there is a 1-in-8 likelihood that a woman will be diagnosed with breast cancer. This is an increase from the previous likelihood of 1-in-11 in the 1970s. Some hypothesize that this greater incidence for breast cancer may be due to both longer life expectancy, varying reproductive behaviors (i.e., delayed childbearing and bearing fewer children), and the greater use of mammography screening (DeSantis et al. 2014). Up until the 1990s, the incidence of breast cancer seemed to be steadily rising. However, this increase began to level off in the 1990s. Furthermore, in the early 2000s there was a drop of about 7% in breast cancer incidence. Researchers believe that this drop was due to a decrease in the use of hormonal treatment for menopause, which was shown to have an association with breast cancer development (DeSantis et al. 2014). According to a particular study, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females across the globe, demonstrating the worldwide relevance of breast cancer (Jemal et al. 1999).

Various Treatments for Breast Cancer

Given the prevalence of breast cancer throughout the population, it is imperative that effective treatment plans are established. There are several options that can be used to treat breast cancer: surgical treatments, radiation, chemotherapy, endocrine therapy, and tissue-targeted therapy. Often times, these treatment options are used in a variety of combinations depending on the stage of the progression of the breast cancer, the type of breast cancer, and the age and pre-existing conditions of the patient. Stage 0 breast cancer, for example, includes lobular carcinoma in situ and ductal carcinoma in situ. For lobular carcinoma, no treatment is required, but the patients with this type of breast cancer should undergo rigorous monitoring as well as pursue preventative endocrine therapeutics, such as tamoxifen, if the cancer is positive for estrogen receptors (Maughan et al. 2010). For ductal carcinoma in situ, the mass should be surgically

removed with breast-conserving surgery and treated with radiation after the surgery. The use of tamoxifen is not recommended in this condition (Maughan et al. 2010). Breast cancer that has progressed to either stage I or stage II, however, requires much more involved treatment plans. In stage I and II, surgical treatment is almost always necessary. Radical mastectomy has been the standard surgical treatment at these stages. However, breast-conserving surgery coupled with radiation therapy has increased in frequency. Given the potential for metastasis at these stages, a lymph node biopsy is usually performed to assess the spread of the cancer (Maughan et al. 2010). Furthermore, chemotherapy, endocrine therapy, and tissue-targeted therapy are all used at these stages of breast cancer. Chemotherapy will almost always be used when the cancer is either node-positive or when there is a tumor larger than 1 centimeter. Chemotherapy is especially necessary to treat those types of cancer that are hormone receptor-negative in nature. For those types of cancer that are hormone receptor-positive, on the other hand, endocrine therapy can be particularly helpful. Given that estrogen promotes the growth of these tumors, the treatments in this category, such as tamoxifen, will either block estrogen production or serve as an estrogen antagonist. Aromatase inhibitors also fall into this category as they prevent the formation of estrogen from androgens (Maughan et al. 2010). Lastly, tissue-targeted therapy involves the use of a monoclonal antibody that will bind the ERBB2 receptor that is overexpressed in some cancer phenotypes (Maughan et al. 2010). For stage III breast cancer diagnoses, which are characterized by tumors larger than 5 centimeters and migration to lymph nodes, induction therapy is necessary. Induction therapy involves the use of chemotherapy to downsize the tumor prior to operation on the tumor. Induction therapy can also be paired with the other treatment options mentioned above (Maughan et al. 2010). Stage IV, or metastatic, breast cancer is very lethal, with a five-year survival rate of only 23.3 percent (Maughan et al. 2010). Treatment of

this stage of cancer is similar to stage I and II cancers: endocrine therapy is used to target hormone receptor-positive cancers, tissue-targeted therapy is used for tumors overexpressing ERBB2, and chemotherapy can be used in many situations. During this stage, which is very lethal, the comfort of the patient must be considered when developing a treatment plan (Maughan et al. 2010). Even with all of the current treatments that exist for breast cancer, there is still a need to seek out new treatments. Effective treatments are those that have high target-tissue specificity; in other words, the goal is to be able to target the cancer cells without harming the healthy cells. Many genes have been associated with the development of cancer, and the use of the CRISPR/Cas9 gene-editing tool may provide a specific method to target cancer cells.

CRISPR/Cas9 as a Gene-Editing Tool

The use of the CRISPR/Cas9 mechanism as a gene-editing tool is a relatively recent development. The process of discovering this gene-editing tool began in 1987, when researchers noticed repetitive sequences at the end of a bacterial gene. A decade later, biologists noticed that these repetitive sequences were actually palindromic in nature and separated by regions of "spacer DNA." Even after the elucidation of these clustered regularly interspaced short palindromic repeats (CRISPR), the function of these sequences was not yet determined. However, in 2005, researchers in the field of bioinformatics discovered that the spacer DNA in between the palindromic repeats matched viral DNA sequences. This led to the conjecture that CRISPR serves as a form of adaptive immunity for bacteria against viruses (Pennisi 2013). Given that bacteria and viruses have coevolved over the years, bacteria have been able to develop a variety of defenses against viruses, such as bacteriophages. Many of the defenses that bacteria have against viruses are innate, but adaptive immunity is also present within bacteria. Danisco, a food science company, was able to demonstrate this adaptive immunity (Klug et al.

2015). Danisco exposed *Streptococcus thermophilus*, a strain of bacteria that is commonly used in the production of both cheese and yogurt, to bacteriophages; exposure of the bacteria to the bacteriophages strengthened the defenses of the bacteria, essentially vaccinating this strain of bacteria against the viruses (Pennisi 2013). The CRISPR/Cas system was determined to be responsible for this adaptive immunity. The CRISPR/Cas system of adaptive immunity operates in three basic steps: acquisition, crRNA biogenesis, and interference (Figure 3). The first step, acquisition, begins when the foreign DNA of the virus is fragmented and inserted into a CRISPR locus. This foreign DNA becomes the "spacer DNA" that is characteristic of these regions. Next, during crRNA biogenesis, the CRISPR locus is transcribed to form crRNA. It is important to mention that various CRISPR-associated (Cas) proteins are needed to process the crRNA that is produced. During the interference stage, which is the last step, the mature crRNAs associate with Cas proteins and bind to the complementary foreign DNA from which the crRNA was derived. Then, the Cas proteins form a double-stranded break within the foreign DNA. It would seem that these CRISPR/Cas complexes would also bind to and inactivate the original CRISPR locus due to complementarity. However, these CRISPR/Cas complexes do not bind to the original CRISPR locus that is present in the bacterial genome, and this is likely due to the CRISPR repeat sequences that identify the sequence as "self" (Klug et al. 2015). The aforementioned mechanism of the CRISPR/Cas system applies to its use within bacteria to provide adaptive immunity. However, this system can be modified to be used in gene-editing. For example, Editas Medicine sought to use the CRISPR/Cas technology to treat loss-of-function and gain-of-function mutations. The mechanism that would be used to treat both of these mutations would involve pairing single guide RNAs (sgRNAs) to the Cas9 endonuclease that produces double-stranded breaks in the target DNA; the sgRNA, through complementary base-pairing, guides the Cas9

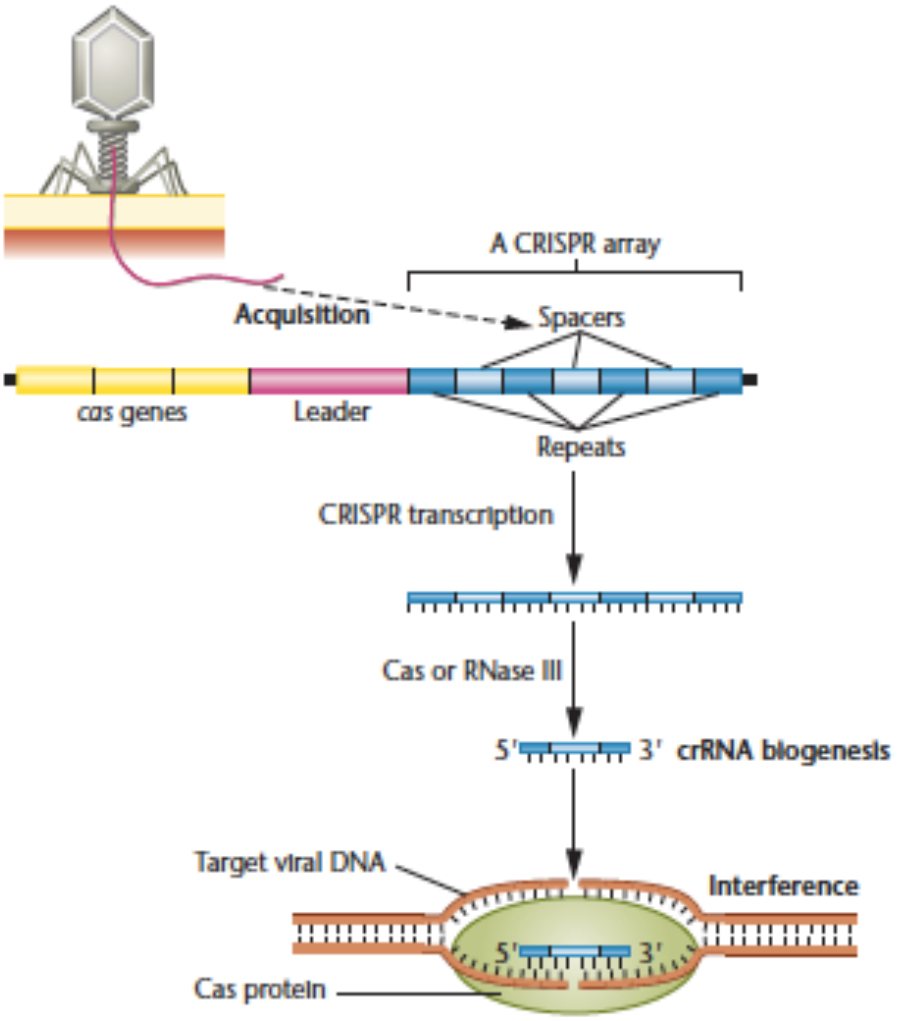

Figure 3: The three basic steps of CRISPR/Cas adaptive immunity in bacteria (Klug et al. 2015).

nuclease to the target sequence. In addition, a sequence must be included that is used as a template during the repair of the double-stranded break. It is this repair of the double-stranded break that impacts the mutation. In recessive disorders, the template sequence that is used for repair is the dominant copy of the gene. In dominant disorders, the template sequence that is used for repair is the recessive gene (Klug et al. 2015). In addition to treating recessive and dominant disorders, CRISPR/Cas9 can also silence the expression of any gene through the same mechanism. The sgRNA, Cas9 protein, and template sequence are usually encoded on a plasmid that is inserted into target cells with a virus. In order to demonstrate the specificity of the CRISPR/Cas9 system, researchers Jennifer Doudna and Emmanuelle Charpentier demonstrated that varying single guide RNAs (sgRNAs) paired with the Cas9 nuclease can form double-stranded breaks in specific sequences of DNA (Pennisi 2013). In addition to having specificity, CRISPR/Cas9 is also more affordable and easier to use than the other forms of gene-editing, such as zinc-finger nucleases and transcription activator-like effector nucleases (TALENs). For example, when using CRISPR/Cas9 in order to target a new site in the genome, only a new sgRNA has to be synthesized; for zinc finger nucleases and TALENs, on the other hand, entirely new proteins have to be synthesized for each newly targeted site within the genome (Xiao-Jie et al. 2015). In addition, Addgene, a biotechnology company, is able to produce CRISPR constructs for any variety of target sequences at an affordable price (Pennisi 2013). Given the capacity of CRISPR/Cas9 to alter the expression of any number of genes, this gene-editing tool can be used to alter those genes that have been explicitly implicated in causing breast cancer. One such gene commonly examined in the development of breast cancer is angiomotin (Amot).

The Angiomotin Target Sequence

Angiogenesis is the formation of new blood vessels from pre-existing vessels; this process occurs during times of growth, such as embryological development. Angiogenesis also plays a significant role in the development of cancer because growing tumors need both nutrients and oxygen from the blood supply. The angiostatin protein is an inhibitor of angiogenesis, and Amot serves as an inhibitor of angiostatin. Therefore, the angiomotin protein activates angiogenesis in tumors (Jiang et al. 2006). There are two isoforms of Amot: Amot80 and Amot130. The two isoforms perform seemingly opposite functions. For example, in a study of retinal angiogenesis in postnatal mice, Amot80 was shown to be clearly expressed during those postnatal days where retinal angiogenesis was expected. After retinal angiogenesis was complete, expression of Amot130 predominated. This demonstrates that the Amot80 isoform is associated with a migratory phenotype for cells that were forming new vessels, while the Amot130 isoform was involved with the maturation and stabilization of the cells of the blood vessels (Ernkvist et al. 2008).

In addition to angiogenesis, both angiomotin isoforms have been found to be involved in the signal cascades responsible for the regulation of organ size. The Hippo pathway is commonly considered to be responsible for controlling cell growth, and it is considered to be a tumor-suppressing pathway. Originally, it was thought that the Hippo pathway proceeds as follows: MST1/2 protein kinases phosphorylate and activate LATS1/2 kinases, which then directly phosphorylate and inactivate the pro-growth transcription factors of YAP and TAZ. However, one study determined that LATS1/2 kinases likely phosphorylate Amot130, which increases the stabilization of this isoform. Then, Amot130 and AIP4 likely form a complex that binds and inhibits YAP. This conclusion was drawn due to the design of the study. For example, wild-type

Amot130 is normally phosphorylated at a serine residue, which enables the protein to eventually inhibit YAP. Growth of cells expressing the wild-type Amot130 was compared to those cells expressing a mutant form of Amot130 that is not able to be phosphorylated at this particular residue. The colonies of cells expressing wild-type Amot130, which was able to inhibit YAP, were consequently much smaller than the colonies expressing a mutant form of Amot130, which was not able to inhibit YAP. This demonstrates that functional Amot130 is necessary to prevent pro-growth conditions in cells (Adler, Johnson, et al. 2013). The aforementioned association between Amot130 and AIP4 was elucidated in a separate study that determined that significant reduction in cell-growth was only seen in those cells that expressed both AIP4 and Amot130 (Adler, Heller, et al. 2013). Although the Amot130 isoform is involved with growth arrest, the Amot80 isoform is involved in cellular proliferation. In particular, Amot80 promotes the proliferation of mammary epithelial cells. Given that breast cancers originate from the proliferation of the epithelial cells of either the ducts or lobules of the breast tissue, it is evident that the gene coding for the Amot80 isoform could be a potential target for treatment. In mature tissues that have an intact apical domain, growth arrest is important. It has been shown that Amot80 can disrupt apical intercellular junctions; this is consistent with the aforementioned finding that Amot80 is associated with the migratory phenotype in angiogenesis. One study found that the mechanism by which Amot80 promotes the proliferation of mammary epithelia is through the increased phosphorylation of extracellular signal-regulated kinases (ERK1/2). It is thought that Amot80 promotes the activation of Raf by Ras; then, activated Raf eventually activates ERK1/2 (Ranahan et al. 2011). In this same study, mammary cells were grown on Matrigel, which simulates the extracellular matrix. Normally, non-transformed MCF10A cells grown on the Matrigel will form hollow, fluid-filled ducts that resemble mammary ducts. The

colonies of MCF10A cells that were expressing Amot80 at higher levels were larger than the control colonies, and they lacked a hollow lumen (Ranahan et al. 2011). The increased proliferation of MCF10A cells expressing Amot80 is a significant finding, especially considering that MCF10A cells are non-tumorigenic in nature. This study alone warrants further investigation into the role of Amot80 in breast cancer.

It has been demonstrated in many studies that the Amot80 isoform of the angiomotin (Amot) gene is strongly associated with angiogenesis and the proliferation of mammary epithelia. Furthermore, it has been discussed that the CRISPR/Cas9 gene-editing tool is both specific and efficient. Therefore, it is reasonable that CRISPR/Cas9 could be used to reduce the expression of Amot in tumorigenic cell lines. The AMOT CRISPRa sgRNA lentivector (set of three targets) constructs will be used to attempt to reduce Amot expression in tumorigenic MDA-MB-468 mammary epithelial cells. It is hypothesized that these constructs will significantly reduce angiomotin expression, as confirmed by quantitative PCR of the cDNA generated from the mRNA of the treated cells.

Materials and Methods

Mammalian Tissue Culture

1. One vial (approximately 1 mL) of MDA-MB-468 human mammary epithelial cells was taken out of the -80 ℃ freezer.
2. As the cells were thawing, 10 mL of properly prepared Leibovitz-15 (L-15) media (10% FBS and 1% antibiotic/antimycotic) was transferred to a 10-cm tissue culture plate.
3. Before the cells thawed entirely, the cells were poured from the vial into the 10-cm tissue culture plate in a sterile tissue culture hood.
4. After the cells were added to the media, the plate was gently moved in a forward-backward, side-to-side, and circular motion to distribute the cells across the plate.
5. The MDA-MB-468 cells were transferred to a 37 ℃ and 0% CO_2 incubator.
6. The next day, the cells were examined to determine their health, and the old media was aspirated off of the plate without disturbing the cells on the plate.
7. 10 mL of fresh Leibovitz-15 media was gently added to the plate, and the cells were returned to the incubator.
8. Following at least a day after media transfer, the cells were split in a 1:1 ratio onto another 10-cm plate. (Note: the ratio at which the cells were split varies according to confluency of the cells on the plate.)
9. To begin the splitting process, the tissue culture hood was prepared and sterilized, the aspirator line was flushed, and all necessary materials were sterilized before being placed in the hood.

10. After preparations were made for the process, the media was carefully aspirated off of the cells.
11. 1 mL of PBS was added to the plate without disrupting the cells, and the PBS was also aspirated off of the plate.
12. Then, 2 mL (volumes can vary depending on the size of the plate) of 1X trypsin was added to the plate, and the plate was returned to the incubator.
13. The plate was gently agitated every 2 to 5 minutes until all of the cells were in suspension.
14. An equal volume of total media was added to the plate at a 45º angle in order to neutralize the trypsin. (Note: the plate was kept at this angle in order to keep the cells from adhering to the base of the plate again.)
15. The total volume (4 mL in this case) was transferred to a 15-mL falcon.
16. The falcon was spun at 1500 xg for 1.5 minutes.
17. A new 10-cm plate (or any other plate size) was labeled with the cell type, the passage number, the researcher's initials, and the date.
18. 10 mL of fresh Leibovitz-15 media was added to the new plate.
19. The supernatant in the 15-mL falcon was aspirated off without disrupting the pellet of cells that was in the bottom of the falcon.
20. Using a P1000 pipet, 1 mL of Leibovitz-15 media was used to resuspend the pellet (the pellet was broken up by pipetting up and down at least 40 times).
21. The resuspended cells were pipetted onto the new plate.
22. This plate was moved in a forward-backward, side-to-side, and a circular motion to evenly distribute the cells on the plate.

23. The plate was transferred to a 37 ℃ and 0% CO_2 incubator.

Pouring LB/Ampicillin Plates

1. A 500-mL glass bottle was selected in order to prepare 250 mL of the LB/ampicillin agar solution.
2. 5 Lennox tablets, containing LB broth with agar, were added to 250 mL of non-sterilized Millipore water (this was consistent with the order to add 1 tablet per 48.3 mL of water).
3. Using an automatic magnetic stirrer, the tablets were dissolved in the water.
4. Then, the lid for the container was placed on the container loosely, and foil was placed over the lid. Lastly, a piece of autoclave tape was placed on the foil.
5. The container was placed in a plastic tub containing just enough water to cover the bottom of the tub.
6. A 15-minute wet cycle was selected.
7. A 55 ℃ water bath was prepared.
8. After the wet cycle was complete, the container of autoclaved LB/agar broth was transferred to the water bath.
9. The temperature of the broth was periodically checked with a sterilized thermometer, until the temperature of the broth was at about 55 ℃.
10. While waiting for the LB/agar solution to cool, twenty 10-cm plates were prepared in a sterile hood; each plate was labelled as “LB/amp” with the date.
11. When the temperature cooled to the aforementioned temperature, 250 µL of the 1000X ampicillin solution was transferred to the LB/agarose solution.
12. The LB/agar solution was swirled in order to mix the ampicillin antibiotic in the LB/agar solution.

13. Enough (approximately 10 mL) of the LB/agar/ampicillin solution was added to each plate in order to cover the bottom of the plate.
14. After all of the plates were poured, the plates were cooled until solid. (Note: the lids were propped open to prevent condensation build-up.)
15. The plates were transferred back into the original bag, the bag was labelled appropriately, and the bag was placed in the refrigerator for storage.

DH5α Transformation

1. One tube of DH5α cells was removed from the -80 ℃ freezer and thawed on ice.
2. The vial containing Scrambled sgRNA CRISPR/Cas9 All-in-one Lentivector was removed from the -4 ℃ freezer and placed in ice to thaw; the three vials containing the three constructs of AMOT CRISPRa sgRNA lentivector (set of three targets) were also removed from the same freezer and placed in ice to thaw.
3. Four 1.5-mL microcentrifuge tubes were assigned one of the following labels: CNTRL (for Scrambled CRISPR), Red (for Amot 98), Yellow (for Amot 174), and Green (for Amot 257).
4. After the DH5α cells thawed, a 50-µL volume of cells was transferred into each of the four microcentrifuge tubes.
5. The unused cells were placed back into the -80 ℃ freezer.
6. 1 µL of each DNA construct was added to the properly labelled tube, and the DNA constructs and cells were gently swirled with the pipet tip.
7. The tubes were incubated on ice for 30 minutes.
8. Each tube of cells was heat shocked for 20 seconds without shaking on a heat block set to 42 ℃.

9. The tubes were incubated on ice again for 2 minutes.
10. 950 µL of pre-warmed SOC media was transferred to each tube.
11. The tubes were then incubated in a shaking incubator at 37 ℃ for 1 hour at 225 rpm.
12. Three LB/ampicillin plates were selected per construct and were labelled with the construct and the amount of volume pipetted onto the plate (either 50 µL, 100 µL, or 300 µL).
13. For each construct, the volumes specified by each plate were pipetted onto the plate and were spread with a sterile inoculation loop in a manner that dilutes the colonies across the plate.
14. The plates were flipped upside down to prevent the accumulation of condensation and were placed in a 37 ℃ incubator overnight.
15. The remaining transformation reactions were stored in the refrigerator indefinitely.
16. The next day, 50 mL of LB/ampicillin broth was prepared with the same concentration of ampicillin (1 µL of ampicillin/1 mL of LB broth) as the plates.
17. Four 3-mL tubes were filled with 3 mL of the LB/ampicillin broth and were labelled for each construct.
18. Colonies were transferred from the plates of each construct into the corresponding tubes of LB/ampicillin broth.
19. The tubes containing the inoculated broth were placed into the shaking incubator that was set to 37 ℃ and 225 rpm for 18 hours.
20. The tubes were inspected for growth.

Bacterial Plasmid DNA "Mini-Prep"

1. The tubes of broth were inspected for growth, and one tube with significant bacterial growth was selected per construct.
2. 1.5 mL of the broth was transferred to the appropriately labeled 2-mL microcentrifuge tube. This was repeated for each construct.
3. The tubes were centrifuged at 15,000 xg for 3 minutes.
4. The supernatant was removed, leaving a pellet at the bottom of the tube.
5. Steps 2-4 were repeated to use the remaining volume of the selected liquid cultures.
6. The pellets of bacteria were then stored in the -4 ℃ freezer.
7. 50 mL of solution 1 (TE Resuspension Buffer) was prepared by adding 2.5 mL of 0.5 M Tris at a pH of 8.0, 500 µL of 1 M EDTA at a pH of 8.0, 0.45 g of glucose, 100 µL of a 50 mg/mL stock solution of Rnase, and a volume of distilled water that makes the total volume 50 mL. Solution 1 was stored in the 4 ℃ refrigerator.
8. 50 mL of solution 2 (NaOH/SDS Lysis Buffer) was prepared by adding 0.5 g of SDS powder to make the concentration 1% and by adding 10 mL of 1 M NaOH to make the final concentration of NaOH 0.2 M. Distilled water was added to make the total volume 50 mL.
9. 50 mL of solution 3 (KOAc Neutralization Buffer) was prepared by adding 50 mL of 5 M potassium acetate to a 50-mL falcon; additionally, glacial acetic acid was added until the pH of the solution was 4.8. This solution was stored at room temperature.
10. The microcentrifuge tubes containing the pellets of bacterial cells transformed by each construct were removed from the -4 ℃ freezer.

11. 200 µL of solution 1 was added to the pellet of each tube, and the pellet was resuspended by pipetting up and down.
12. 200 µL of solution 2 was added to each tube, and the tubes were inverted several times to mix. The samples were assessed to ensure that each sample was clear at this point.
13. 200 µL of solution 3 was added to each tube, and the tubes were inverted to mix. The samples were assessed to ensure that each sample had a white precipitate.
14. The tubes were centrifuged at 15,000 xg for 5 minutes in the mini centrifuge.
15. The supernatant was removed from each tube and transferred to a new tube that was properly labeled. The tubes containing white precipitate were discarded.
16. Each tube was inspected for any particulates; if any particulates were present, steps 14 and 15 were repeated.
17. After ensuring there were no particulates in the new tubes, 1 mL of 100% ethanol was added to each tube.
18. The tubes were centrifuged at 15,000 xg for 5 minutes in the mini centrifuge.
19. The ethanol was removed, making sure not to disrupt the pellet in the process.
20. 1 mL of 70% ethanol was added to each tube; the pellet of each tube was dislodged in this step.
21. The tubes were centrifuged at 15,000 xg for 2 minutes in the mini centrifuge.
22. All of the ethanol was removed by pipetting progressively smaller volumes of ethanol off of the pellet of each tube.
23. Once all of the ethanol was removed, the pellet of each tube was allowed to air dry for 5-10 minutes.
24. Each pellet was resuspended with 50 µL of TE solution.

25. The optical density and concentration of each DNA sample were determined through the use of the NanoDrop Lite Spectrophotometer.
26. The prepared DNA was stored in the 4 ℃ refrigerator until needed for transfections.

Bacterial Glycerol Stock Preparation

1. Eight cryovials were selected, and two cryovials were designated for each construct; for example, two cryovials were labeled with Scrambled CRISPR, Amot 98, Amot 174, or Amot 257.
2. 500 µL of 50% glycerol was added to each cryovial.
3. 500 µL of the liquid culture transformed with the Scrambled CRISPR plasmid was added to the corresponding cryovials. The liquid culture was resuspended by pipetting up and down prior to adding liquid culture to the cryovial.
4. Step 3 was repeated for each CRISPR construct (i.e., Amot 98, Amot 174, and Amot 257).
5. Once all of the glycerol stocks were prepared, the eight cryovials were put into the -80 ℃ freezer for long-term storage of the bacteria transformed with the desired DNA constructs.

6-Well Plate Cell Assay

1. After the DNA was prepared, the MDA-MB-468 cells that were in culture were inspected for adequate growth.
2. Once the MDA-MB-468 cells were determined to be at a confluency (approximately 70% confluent) that was sufficient for a 6-well plate cell assay, preliminary calculations were performed to prepare the two 6-well plates that were needed for the experiment.

3. It was determined that 600,000 cells should be added to each well.
4. The calculation for total cells needed was performed by multiplying the 600,000 cells/well by 13 wells (to provide extra cells to prevent error); this yielded a total of 7,800,000 cells.
5. The total volume of media that will be added to the two 6-well plates was determined to be 26 mL (2 mL of media per well x 13 wells).
6. After these preliminary calculations, two 10-cm plates of MDA-MB-468 cells were selected to be used in the 6-well plate assay.
7. To begin the process, the cells were split using normal tissue culture techniques.
8. The old media was aspirated off of each plate.
9. 1 mL of 1X PBS was added to each plate, and the PBS solution was then aspirated off of each plate.
10. Then, 2 mL of 1X trypsin was added to each plate, and the plates were placed into the 37 ℃ incubator for 8 to 12 minutes. The plates were gently agitated and examined every 2-5 minutes to determine if the cells were detached from the plate.
11. After all cells were detached from the plate, an equal volume of L-15 media (2 mL) was added to each plate.
12. The cells from both plates were transferred to a 15-mL falcon and were spun at 1500 xg for 1.5 minutes.
13. The media was carefully aspirated off of the pellet, and the pellet was resuspended with 1 mL of L-15 media.

14. Immediately after resuspension, 10 µL of the suspended cell solution was added to a 10-µL drop of trypan blue that was placed on a piece of parafilm. The cells were mixed with the trypan blue by pipetting up and down.
15. After mixing the cells with the trypan blue, 10 µL of the stained cells was added to the opening of a cell counter slide at a 45º angle. The cell counter slide was inserted into the Bio-Rad Automated Cell Counter, and the cell count was too high for the Automated Cell Counter to determine the exact cell count.
16. The resuspended cells were resuspended with an additional 1 mL of L-15 media, and the same process was repeated as described in steps 14 and 15.
17. The live cell count was determined to be 6.78×10^6 cells/mL.
18. After determining the cell count, it was determined that 1,150 µL of the resuspended solution of cells would be needed to provide the 7,800,000 cells for the two 6-well plate assays.
19. A 50-mL falcon was acquired, and 26 mL of L-15 media was added to the falcon.
20. Then, the solution of cells was thoroughly resuspended, and 1 mL (or 1000 µL) of the resuspended solution was added to the falcon containing the L-15 media. The solution of cells was resuspended again, and the remaining 150 µL of the resuspended cell solution was added to the falcon containing the L-15 media.
21. The two 6-well plates were acquired and labeled as follows: the first plate was labeled with “CNTRL” for row A and “Amot 98” for row B, and the second plate was labeled with “Amot 174” for row A and “Amot 257” for row B. In addition, the plates were labeled with the experimenter’s initials, the passage number of the cells, and the date.

22. The suspension of cells was inverted to mix the cells, and a pipet aid with a 10-mL pipet tip was used to collect 6 mL of the resuspended cell solution. Then, 2 mL of the resuspended cell solution was added to each well of the first row.
23. Step 22 was repeated for the other 3 rows.
24. After 2 mL of the resuspended cell solution was added to each well, the two 6-well plates were added to the 37℃ and 0% CO_2 incubator.
25. The remaining volumes of cell suspension in the 15-mL falcon and the 50-mL falcon were combined and added to two 10-cm tissue culture plates containing fresh L-15 media. These plates were also placed in the 37℃ and 0% CO_2 incubator.

Transient Transfection Protocol

1. After 24 hours, the 6-well plates were inspected to determine if there were enough cells present for transfection.
2. The L-15 media was aspirated off of the cells in each well, and 1 mL of Opti-MEM was transferred to each well.
3. The 6-well plates were placed in the 37 ℃ and 0% CO_2 incubator while the remainder of the transfection protocol was taking place.
4. It was previously determined that each construct would be tested at three different concentrations: 1 µg, 2 µg, and 4 µg.
5. Given the varying concentrations of each DNA construct, calculations were performed to determine what volume of each DNA construct would have to be added to yield the desired concentration of DNA.
6. After these calculations were performed, 15 1.5-mL microcentrifuge tubes were acquired; twelve tubes were set aside for the preparation of the DNA, and three tubes were set aside

for the preparation of the transfectin reagent. Each set of three 1.5-mL microcentrifuge tubes were labeled with the appropriate construct and the concentration (either 1 µg, 2 µg, or 4 µg). The remaining three tubes were labeled with "transfectin" and one of three volumes of the reagent: 2 µL, 4 µL, or 8 µL.

7. The concentrations of each DNA construct were used to determine how much of the solution containing the DNA needed to be added to provide 1 µg, 2 µg, or 4 µg of the DNA.
8. Prior to adding any DNA to any tubes, 250 µL of Opti-MEM was added to each of the twelve tubes.
9. For the Scrambled CRISPR construct, 2.4 µL of the DNA was added to the 1-µg tube, 4.9 µL was added to the 2-µg tube, and 9.7 µL was added to the 4-µg tube.
10. For the Amot 98 construct, 1.95 µL of the DNA was added to the 1-µg tube, 3.9 µL was added to the 2-µg tube, and 7.8 µL was added to the 4-µg tube.
11. For the Amot 174 construct, 1.4 µL of the DNA was added to the 1-µg tube, 2.8 µL was added to the 2-µg tube, and 5.6 µL was added to the 4-µg tube.
12. For the Amot 257 construct, 3.7 µL of the DNA was added to the 1-µg tube, 7.3 µL was added to the 2-µg tube, and 14.6 µL was added to the 4-µg tube.
13. Given that 2 µL of transfectin needs to be added to each tube of 1 µg of DNA, one large aliquot was prepared. This aliquot was formed by adding 10 µL of transfectin to 1,250 µL of Opti-MEM. (Note: A 5X aliquot was made to ensure that there was extra solution available.)

14. Given that 4 µL of transfectin needs to be added to each tube of 2 µg of DNA, one large aliquot was prepared. This aliquot was formed by adding 20 µL of transfectin to 1,250 µL of Opti-MEM.
15. Given that 8 µL of transfectin needs to be added to each tube of 4 µg of DNA, one large aliquot was prepared. This aliquot was formed by adding 40 µL of transfectin to 1,250 µL of Opti-MEM.
16. After the aliquots of transfectin were prepared, 250 µL of the 2-µL transfectin aliquot was added to the 1-µg tube of Scrambled CRISPR DNA. Then, 250 µL of the 4-µL transfectin aliquot was added to the 2-µg tube of Scrambled CRISPR DNA. Finally, 250 µL of the 8-µL transfectin aliquot was added to the 4-µg tube of Scrambled CRISPR DNA.
17. The same volumes of each transfectin aliquot were added to each concentration of DNA for the other three constructs.
18. The tubes containing both the DNA and the transfectin were allowed to incubate at room temperature for 20 minutes.
19. After this incubation period, the entire volume of the solution containing 1 µg of Scrambled CRISPR DNA and 2 µL of transfectin was added to the well that corresponds to 1 µg of the Scrambled CRISPR DNA. This solution was added dropwise to the solution while the plate was tilted; after adding a few drops, the plate would be moved forward and backward to distribute the DNA equally.
20. Step 19 was repeated for each construct at each concentration. (Note: Special care was taken to prevent cross-contamination of DNA in different wells.)
21. The 6-well plates were then placed in the 37 ℃ and 0% CO_2 incubator.

22. After six hours of incubation, 1 mL of Opti-MEM was added to each well.
23. The 6-well plates were inspected at 24 hours, 48 hours, and 72 hours of incubation.
24. At 48 hours, the 4-µg wells for each construct were harvested.
25. To harvest the cells, the media was aspirated off of each well, and 500 µL of Trizol was added to the cell culture in each well. (Note: Special precautions were taken when Trizol was in use.)
26. The viscous solution that resulted from the addition of the Trizol solution was transferred to a 1.5-mL tube that was labeled with the proper DNA construct (i.e., Scrambled, Amot 98, Amot 174, or Amot 257) along with the concentration of the DNA (1 µg, 2 µg, or 4 µg). These tubes could be stored in a -20 ℃ freezer until needed for an RNA extraction.
27. At 72 hours, the 1-µg and 2-µg conditions were both harvested by repeating steps 25 and 26.
28. All of the tubes were stored in the -20 ℃ freezer until needed for the RNA extraction.

RNA Extraction

1. Prior to beginning the RNA extraction process, the tissue culture hood was prepared by spraying RNAse-away on all surfaces that would be touched throughout the process.
2. A bucket of ice was collected and wiped down with RNAse-away before being inserted into the culture hood.
3. The tubes containing the harvested cells were removed from the freezer, wiped down with RNAse away, and allowed to thaw.
4. While the tubes were thawing, the refrigerated centrifuge was turned on and set to 13,000 xg and 4 ℃.

5. After the tubes were able to incubate at room temperature for about 5 minutes, 0.2 mL of chloroform was added to each tube containing Trizol.
6. After adding chloroform to each tube, the tubes were vortexed and incubated at room temperature for 5 minutes.
7. After this room temperature incubation, the tubes were centrifuged at 13,000 xg for 10 minutes at 4 ℃. After this, the tubes were immediately put on ice. (Note: the tubes were kept on ice at all times unless a specific room temperature incubation was required.)
8. The top layer (aqueous layer) of each tube was transferred to a new tube that was labeled appropriately. (Note: if any contamination occurred, the aqueous layer was pipetted back into the tube, and step 9 was followed until the transfer was successful.)
9. The tubes were centrifuged again at 13,000 xg for 3 minutes at 4 ℃ if any contamination occurred (such as the disruption of any of the other layers).
10. Once the aqueous layer of each tube was successfully transferred to a new tube that was appropriately labeled, 500 µL of isopropanol was added to each tube.
11. After adding isopropanol to each tube, 0.5 µL of glycoblue was added to each tube. (Note: the glycoblue was pipetted directly into the liquid layer.)
12. After the addition of isopropanol and glycoblue, the tubes were vortexed, incubated at room temperature for 5 minutes, and centrifuged at 13,000 xg for 10 minutes at 4 ℃.
13. The isopropanol was removed from each tube without disrupting the blue pellet at the bottom of each tube.
14. After removing as much isopropanol as possible from each tube, 500 µL of 75% ethanol was added to each tube. (Note: the pellet was purposefully dislodged at this step.)
15. The tubes were spun at 13,000 xg for 5 minutes at 4 ℃.

16. The ethanol was carefully removed from each pellet through the successive use of adjustable pipets with smaller volume capacities (i.e., P1000 to P200 to P10 to P2).
17. With just a miniscule amount of ethanol left in the bottom of the tube, the tubes were spun in a table-top centrifuge to spin down any remaining ethanol. A P2 pipet was then used to remove the last bit of ethanol.
18. The tubes were left open for 5 minutes to allow the pellet to dry. (Note: care was taken to prevent any ice from getting into the open tubes.)
19. After all of the ethanol was removed from each tube and each pellet was allowed to dry for about 5 minutes, the pellet was resuspended with 20-50 µL of nuclease free water. (Note: the pellet was mixed by pipetting up and down at least 100 times.)
20. The optical density and concentration of each sample was determined through the use of the NanoDrop Lite Spectrophotometer. The optical density and concentration of each sample was written on the tube.
21. The tubes containing the RNA were placed in the -20 ℃ freezer until cDNA needed to be generated.

cDNA Synthesis

1. The tissue culture hood was prepared by wiping down all surfaces with RNAse-away.
2. The RNA samples were removed from the -20 ℃ freezer, wiped off with RNAse-away, and put on ice to thaw. The 5x iScript advance reaction mix and iScript advanced reverse transcriptase was acquired and put on ice to thaw. Finally, an aliquot of nuclease-free water was acquired and put on ice.
3. Calculations were performed to determine the exact amount of RNA template that needed to be added to a PCR tube to yield 1 µg of total RNA.

4. Instead of using individual PCR tubes for the cDNA synthesis, the top row of a 96-well plate was used.
5. The reagents for cDNA synthesis were added in the following order: nuclease-free water, RNA template, reaction mix, and then reverse transcriptase.
6. The 1 µg, 2 µg, and 4 µg samples of Scrambled CRISPR (CNTRL) were assigned to wells A1, A2, and A3, respectively. The three samples of Amot 98 were assigned in the same order to wells A4, A5, and A6; the three samples of Amot 174 were assigned to wells A7, A8, and A9; and the three samples of Amot 257 were assigned to wells A10, A11, and A12.
7. In A1, 12.5 µL of nuclease-free water, 2.5 µL of CNTRL 1 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
8. In A2, 1.8 µL of nuclease-free water, 13.2 µL of CNTRL 2 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
9. In A3, 2.4 µL of nuclease-free water, 12.6 µL of CNTRL 4 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
10. In A4, 13.3 µL of nuclease-free water, 1.7 µL of Amot 98 1 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
11. In A5, 1.7 µL of nuclease-free water, 13.3 µL of Amot 98 2 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
12. In A6, 9.6 µL of nuclease-free water, 5.4 µL of Amot 98 4 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
13. In A7, 12.8 µL of nuclease-free water, 2.2 µL of Amot 174 1 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.

14. In A8, 0 µL of nuclease-free water, 21.8 µL of Amot 174 2 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
15. In A9, 4.9 µL of nuclease-free water, 10.1 µL of Amot 174 4 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
16. In A10, 13.1 µL of nuclease-free water, 1.9 µL of Amot 257 1 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
17. In A11, 4.1 µL of nuclease-free water, 10.1 µL of Amot 257 2 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
18. In A12, 9.6 µL of nuclease-free water, 5.4 µL of Amot 257 4 µg RNA template, 4 µL of reaction mix, and 1 µL of reverse transcriptase were added to the bottom of the well.
19. The 96-well plate was sealed tightly with the plastic cover for the plate, and the 96-well plate was put into the Bio-Rad CFX96 Real-Time System thermal cycler. The parameters were programmed as follows: 30 minutes at 42 ℃ and 5 minutes at 85 ℃.
20. The cDNA was stored in the 4 ℃ refrigerator until needed for qPCR.

Quantitative PCR (qPCR)

1. It was decided that three 96-well plates would be set up: one for the 1-µg condition of each construct, one for the 2-µg condition of each construct, and one for the 4-µg condition of each construct.
2. In each plate, the general layout was the same. In rows A and E, the target was ß-actin; in rows B and F, the target was GAPDH; in rows C and G, the target was Amot; and in rows D and H, the target was PCNA.
3. The first quadrant of each plate (A1-A4, B1-B4, C1-C4, D1-D4) was designed to be for the CNTRL samples.

4. The second quadrant of each plate (E1-E4, F1-F4, G1-G4, H1-H4) was designed to be for the Amot 98 samples.
5. The third quadrant of each plate (A6-A9, B6-B9, C6-C9, D6-D9) was designed to be for the Amot 174 samples.
6. The fourth quadrant of each plate (E6-E9, F6-F9, G6-G9, H6-H9) was designed to be for the Amot 257 samples.
7. The no-template controls for each plate were designated for wells G11, G12, H11, and H12.
8. After designing the layout of each plate, the primer master mixes were prepared.
9. The forward and reverse primers for each target, SYBR green super mix, and double-distilled water (ddH_2O) were acquired and put on ice in the tissue culture hood.
10. Four 1.5-mL microcentrifuge tubes were acquired and labeled with one of the four targets.
11. The ß-actin master mix was prepared by adding 52 µL of ddH_2O, 28 µL of forward and reverse ß-actin primers (at 3µM concentration), and 100 µL of the SYBR green super mix to a 1.5-mL tube. The master mix was kept on ice after preparation. (Note: the primer master mixes were prepared at 20X instead of 1X.)
12. The GAPDH master mix was prepared by adding 52 µL of ddH_2O, 28 µL of forward and reverse GAPDH primers (at 3µM concentration), and 100 µL of the SYBR green super mix to a 1.5-mL tube. The master mix was kept on ice after preparation.
13. The Amot master mix was prepared by adding 52 µL of ddH_2O, 28 µL of forward and reverse Amot primers (at 3µM concentration), and 100 µL of the SYBR green super mix to a 1.5-mL tube. The master mix was kept on ice after preparation.

14. The PCNA master mix was prepared by adding 52 µL of ddH_2O, 28 µL of forward and reverse PCNA primers (at 3µM concentration), and 100 µL of the SYBR green super mix to a 1.5-mL tube. The master mix was kept on ice after preparation.
15. The no-template controls were prepared as a 5X master mix. For example, 20 µL of ddH_2O, 5 µL of forward and reverse ß-actin primers (at 3 µM concentration), and 25 µL of the SYBR green super mix were added to a 1.5-mL tube.
16. 10 µL of the no-template control master mix was added to wells G11, G12, H11, and H12. This section of wells was kept covered by the plastic cover when adding the primer master mix and the cDNA samples to the 96-well plate.
17. 9 µL of the ß-actin primer mix was added to wells A1-A9 (column 5 was excluded for every row) and wells E1-E9.
18. 9 µL of the GAPDH primer mix was added to wells B1-B9 and wells F1-F9.
19. 9 µL of the Amot primer mix was added to wells C1-C9 and wells G1-G9.
20. 9 µL of the PCNA primer mix was added to wells D1-D9 and wells H1-H9.
21. After all of the primers were loaded, the primers, SYBR green super mix, and the ddH_2O were placed back into the -4 ℃ freezer, the -20 ℃ freezer, and the 4 ℃ refrigerator, respectively.
22. The 96-well plate was removed from the 4 ℃ refrigerator and placed in the tissue culture hood.
23. 1 µL of the CNTRL 1 µg sample was loaded directly into each well containing primer mix in the first quadrant. (Note: the pipet tip was changed for each well.)
24. 1 µL of the Amot 98 1 µg sample was loaded directly into each well containing primer mix in the second quadrant.

25. 1 µL of the Amot 174 1 µg sample was loaded directly into each well containing primer mix in the third quadrant.
26. 1 µL of the Amot 257 1 µg sample was loaded directly into each well containing primer mix in the fourth quadrant.
27. The fully prepared 96-well plate was sealed tightly with the adhesive cover; after sealing, the plate was vortexed and centrifuged.
28. The plate was inserted into the Bio-Rad CFX96 Real-Time System with well A1 in the top left corner.
29. The program entitled "Universal iScript Advanced" was selected on the thermal cycler.
30. The specific parameters of this program were programmed as follows: 1 cycle of 95 ℃ for 30 seconds; 35-40 cycles of 95 ℃ for 10 seconds and 60 ℃ for 20 seconds; and a melt-curve analysis that proceeded from 65 ℃ to 95 ℃ in 0.5 ℃ increments (image capturing occurred every 0.5 ℃).
31. The raw data was collected from the CFX96 Real-Time System and analyzed with the Bio-Rad CFX Maestro program.
32. Steps 9-31 were repeated for the 2-ug and 4-ug samples.

Results

After performing the bacterial plasmid DNA "mini-prep," the optical density and concentration were determined for each construct (Table 1). The A260/280 reading is the

Table 1: Optical density and concentration for each CRISPR construct

Construct	A260/280	Concentration
Scrambled CRISPR (CNTRL)	1.95	411.9 ng/µL
Amot 98	2.00	513.0 ng/µL
Amot 174	2.00	712.9 ng/µL
Amot 257	1.99	273.4 ng/µL

"optical density" of each construct. It was apparent that each construct had a A260/280 value that was either exactly 2.00 or extremely close to that value. A value of 2.00 for the A260/280 reading is ideal for a pure DNA sample. This DNA was used to transfect the MDA-MB-468 cells at varying concentrations. For the 4-µg condition, the cells were harvested after 48 hours of transient transfection due to significant deterioration of the health of the cells; for the 1-µg and the 2-µg conditions, however, the cells were able to be incubated for 72 hours before harvesting the cells. After harvesting the cells at each condition, an RNA extraction was performed. After performing the RNA extraction, the optical density and concentration were determined for each sample (Table 2). Similar to a DNA sample, a value of 2.00 for the A260/280 is considered pure for RNA samples. Each RNA sample had a value close to 2.00 for the A260/280 reading (Table 2). In addition, the concentration of each RNA sample was sufficient for a cDNA preparation. After the generation of cDNA, quantitative polymerase chain reaction (qPCR) was performed to

Table 2: Optical density and concentration for each RNA sample

Sample	A260/280	Concentration (ng/µL)
CNTRL 1 µg	1.91	404.3 ng/µL
Amot 98 1 µg	1.98	573.2 ng/µL
Amot 174 1 µg	1.98	451.6 ng/µL
Amot 257 1 µg	1.96	535.2 ng/µL
CNTRL 2 µg	1.95	75.7 ng/µL
Amot 98 2 µg	1.94	75.4 ng/µL
Amot 174 2 µg	1.92	45.9 ng/µL
Amot 257 2 µg	1.94	91.6 ng/µL
CNTRL 4 µg	1.88	79.4 ng/µL
Amot 98 4 µg	1.96	186.8 ng/µL
Amot 174 4 µg	1.96	98.7 ng/µL
Amot 257 4 µg	1.99	186.4 ng/µL

analyze relative expression of the target genes of angiomotin (Amot) and proliferating cell nuclear antigen (PCNA) in each sample.

Quantitative PCR was performed for each condition: the 1-µg condition, the 2-µg condition, and the 4-µg condition. Within each condition, the impacts of the Amot 98, Amot 174, and Amot 257 CRISPR constructs on the relative gene expression of the aforementioned targets were compared to those of Scrambled CRISPR, the control sample. In the 1-µg condition, each cDNA sample was targeted for expression of ß-actin, GAPDH, Amot, and PCNA, and the Cq values were determined through the use of the Bio-Rad CFX96 Real-Time System (Table 3). Each target was performed in quadruplicate, so the mean Cq values were important in the eventual calculation of ΔΔCq. For the Scrambled CRISPR (or CNTRL) sample, the mean Cq value for ß-actin was 16.24; the mean Cq value for GAPDH was 15.86; the mean Cq value for Amot was 20.05; and the mean Cq value for PCNA was 17.48. With the targets in the same order for the Amot 98 samples, the mean Cq values were determined to be 16.36, 15.84, 19.75, and 17.07. For the Amot 174 samples, the mean Cq values were determined to be 16.30, 15.86, 20.35, and 17.73. Lastly, for the Amot 257 samples, the mean Cq values were determined to be 15.74, 15.52, 19.46, and 17.08. In the no-template control samples, the mean Cq value was determined to be 38.09. In addition to simply determining the Cq values for each sample, melt temperature analysis was performed (Table 3). For each sample targeting ß-actin, the melt temperature was determined to be 89.5 ℃. For each sample targeting GAPDH, the melt temperature was determined to 85 ℃. For each sample targeting Amot, the melt temperature was determined to be 84 ℃. For each sample targeting PCNA, the melt temperature was determined to be 86 ℃. Finally, for the no-template control samples, no melt temperature was determined. The acquisition of the Cq values was important to determining relative normalized expression of

Table 3: Cq values and melt temperatures across the 1-µg condition

Target	Content	Sample	Cq	Melt Temp. (℃)
Actin	Pos Ctrl	Cntrl 1 ug	16.66	89.50
Actin	Pos Ctrl	Cntrl 1 ug	16.09	89.50
Actin	Pos Ctrl	Cntrl 1 ug	15.98	89.50
Actin	Pos Ctrl	Cntrl 1 ug	16.23	89.50
GAPDH	Pos Ctrl	Cntrl 1 ug	15.81	85.50
GAPDH	Pos Ctrl	Cntrl 1 ug	15.86	85.00
GAPDH	Pos Ctrl	Cntrl 1 ug	15.95	85.00
GAPDH	Pos Ctrl	Cntrl 1 ug	15.81	85.50
Amot	Pos Ctrl	Cntrl 1 ug	19.97	84.00
Amot	Pos Ctrl	Cntrl 1 ug	20.03	84.00
Amot	Pos Ctrl	Cntrl 1 ug	20.10	84.00
Amot	Pos Ctrl	Cntrl 1 ug	20.11	84.00
PCNA	Pos Ctrl	Cntrl 1 ug	17.54	86.00
PCNA	Pos Ctrl	Cntrl 1 ug	17.41	86.00
PCNA	Pos Ctrl	Cntrl 1 ug	17.53	86.00
PCNA	Pos Ctrl	Cntrl 1 ug	17.45	86.00
Actin	Unkn	Amot 98 1 ug	16.29	89.50
Actin	Unkn	Amot 98 1 ug	16.09	89.50
Actin	Unkn	Amot 98 1 ug	16.08	89.50
Actin	Unkn	Amot 98 1 ug	16.97	89.50
GAPDH	Unkn	Amot 98 1 ug	15.93	85.00
GAPDH	Unkn	Amot 98 1 ug	15.69	85.00
GAPDH	Unkn	Amot 98 1 ug	15.94	85.00
GAPDH	Unkn	Amot 98 1 ug	15.79	85.00
Amot	Unkn	Amot 98 1 ug	19.73	84.00
Amot	Unkn	Amot 98 1 ug	19.76	84.00
Amot	Unkn	Amot 98 1 ug	19.77	84.00
Amot	Unkn	Amot 98 1 ug	19.75	84.00
PCNA	Unkn	Amot 98 1 ug	17.22	86.00
PCNA	Unkn	Amot 98 1 ug	17.05	86.00
PCNA	Unkn	Amot 98 1 ug	17.05	86.00
PCNA	Unkn	Amot 98 1 ug	16.95	86.00
Actin	Unkn	Amot 174 1 ug	16.39	89.50
Actin	Unkn	Amot 174 1 ug	16.36	89.50
Actin	Unkn	Amot 174 1 ug	16.22	89.50
Actin	Unkn	Amot 174 1 ug	16.24	89.50
GAPDH	Unkn	Amot 174 1 ug	15.74	85.00
GAPDH	Unkn	Amot 174 1 ug	15.84	85.00
GAPDH	Unkn	Amot 174 1 ug	15.96	85.00
GAPDH	Unkn	Amot 174 1 ug	15.92	85.00
Amot	Unkn	Amot 174 1 ug	20.29	84.00
Amot	Unkn	Amot 174 1 ug	20.33	84.00
Amot	Unkn	Amot 174 1 ug	20.46	84.00
Amot	Unkn	Amot 174 1 ug	20.34	84.00
PCNA	Unkn	Amot 174 1 ug	17.66	86.00
PCNA	Unkn	Amot 174 1 ug	17.85	86.50
PCNA	Unkn	Amot 174 1 ug	17.72	86.50
PCNA	Unkn	Amot 174 1 ug	17.68	86.00

Actin	Unkn	Amot 257 1 ug	15.72	89.50
Actin	Unkn	Amot 257 1 ug	15.79	89.50
Actin	Unkn	Amot 257 1 ug	15.86	89.50
Actin	Unkn	Amot 257 1 ug	15.60	89.50
GAPDH	Unkn	Amot 257 1 ug	15.47	85.50
GAPDH	Unkn	Amot 257 1 ug	15.54	85.00
GAPDH	Unkn	Amot 257 1 ug	15.67	85.00
GAPDH	Unkn	Amot 257 1 ug	15.38	85.00
Amot	Unkn	Amot 257 1 ug	19.40	84.00
Amot	Unkn	Amot 257 1 ug	19.48	84.00
Amot	Unkn	Amot 257 1 ug	19.47	84.00
Amot	Unkn	Amot 257 1 ug	19.48	84.00
PCNA	Unkn	Amot 257 1 ug	17.06	86.00
PCNA	Unkn	Amot 257 1 ug	17.06	86.50
PCNA	Unkn	Amot 257 1 ug	17.17	86.50
PCNA	Unkn	Amot 257 1 ug	17.03	86.50
Actin	NTC	N/A	37.71	None
Actin	NTC	N/A	39.51	None
Actin	NTC	N/A	36.52	None
Actin	NTC	N/A	38.63	None

the two target genes (Amot and PCNA) in cells transfected with the treatment CRISPR constructs (Amot 98, Amot 174, and Amot 257). Normalized expression, or ΔΔCq, is calculated by first setting the expression of each target gene (Amot and PCNA) relative to the positive controls (ß-actin and GAPDH) to 1 in the cells transfected with the Scrambled CRISPR control plasmid. Then, the relative expression of the target genes is either higher or lower than 1 in those cells transfected with the Amot 98, Amot 174, or Amot 257 treatment plasmids. The Bio-Rad CFX Maestro software was used to generate the normalized expression of each target in the 1-µg condition (Figure 4). From the normalized expression, it appeared that the expression of Amot and PCNA was significantly less in the cells transfected with 1 µg of the Amot 174 construct than in the cells transfected with the same concentration of the Scrambled CRISPR construct. In those cells transfected with 1 µg of the Amot 98 construct, the expression of Amot and PCNA actually appeared to be significantly higher than the expression within the control group. Finally, in the cells transfected with 1 µg of the Amot 257 construct, the expression of Amot showed a significant increase; PCNA expression, on the other hand, showed no significant increase or decrease.

Quantitative PCR was also performed for the 2-µg condition. Identical to the design of 1-µg condition, each cDNA sample from the cells transfected with 2 µg of DNA was targeted for ß-actin, GAPDH, Amot, and PCNA. The CFX96 Real-Time System was used to generate the Cq values for each particular target within the different cDNA samples (Table 4). For the Scrambled CRISPR (CNTRL) samples, the mean Cq value for the ß-actin target was 33.89; the mean Cq value for the GAPDH target was 21.32; the mean Cq value for the Amot target was 24.70; and the mean Cq value for the PCNA target was 22.66. With the targets listed in the same order, the mean Cq values for the Amot 98 samples were 23.05, 21.27, 24.31, and 22.80, respectively. The

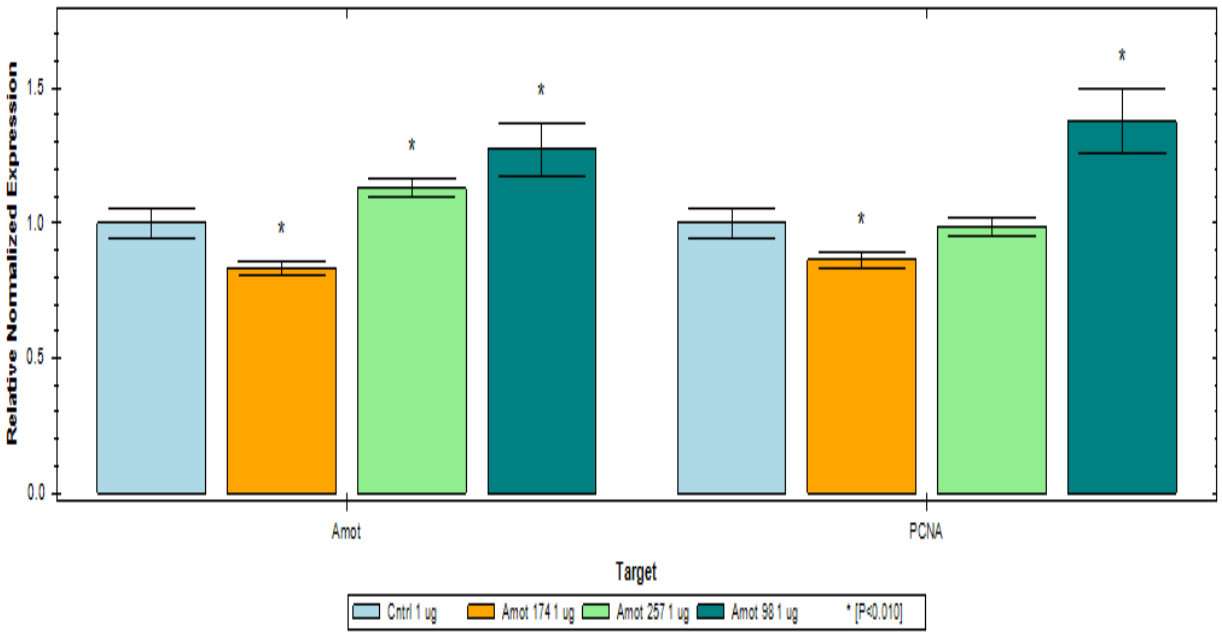

Figure 4: This figure depicts the normalized expression graph for the 1-µg condition. As mentioned, the expression within the control was set to 1.0. The error bars represent standard error of the mean. Significance was determined by a P-value less than 0.01.

Table 4: Cq values and melt temperatures across the 2-µg condition

Target	Content	Sample	Cq	Melt Temp. (℃)
Actin	Pos Ctrl	Cntrl 2 ug	33.61	None
Actin	Pos Ctrl	Cntrl 2 ug	-	None
Actin	Pos Ctrl	Cntrl 2 ug	36.41	None
Actin	Pos Ctrl	Cntrl 2 ug	31.65	88.50
GAPDH	Pos Ctrl	Cntrl 2 ug	21.38	84.50
GAPDH	Pos Ctrl	Cntrl 2 ug	21.25	84.50
GAPDH	Pos Ctrl	Cntrl 2 ug	21.32	84.50
GAPDH	Pos Ctrl	Cntrl 2 ug	21.31	84.50
Amot	Pos Ctrl	Cntrl 2 ug	24.71	83.50
Amot	Pos Ctrl	Cntrl 2 ug	24.70	83.50
Amot	Pos Ctrl	Cntrl 2 ug	24.72	83.50
Amot	Pos Ctrl	Cntrl 2 ug	24.68	83.50
PCNA	Pos Ctrl	Cntrl 2 ug	22.72	86.50
PCNA	Pos Ctrl	Cntrl 2 ug	22.61	86.00
PCNA	Pos Ctrl	Cntrl 2 ug	22.62	86.00
PCNA	Pos Ctrl	Cntrl 2 ug	22.69	86.00
Actin	Unkn	Amot 98 2 ug	23.46	89.00
Actin	Unkn	Amot 98 2 ug	23.09	89.00
Actin	Unkn	Amot 98 2 ug	22.89	89.00
Actin	Unkn	Amot 98 2 ug	22.76	89.00
GAPDH	Unkn	Amot 98 2 ug	21.28	84.50
GAPDH	Unkn	Amot 98 2 ug	21.33	84.50
GAPDH	Unkn	Amot 98 2 ug	21.31	84.50
GAPDH	Unkn	Amot 98 2 ug	21.16	85.00
Amot	Unkn	Amot 98 2 ug	24.26	83.50
Amot	Unkn	Amot 98 2 ug	24.31	83.50
Amot	Unkn	Amot 98 2 ug	24.31	83.50
Amot	Unkn	Amot 98 2 ug	24.38	83.50
PCNA	Unkn	Amot 98 2 ug	22.87	86.50
PCNA	Unkn	Amot 98 2 ug	22.73	86.00
PCNA	Unkn	Amot 98 2 ug	22.80	86.00
PCNA	Unkn	Amot 98 2 ug	22.80	86.00
Actin	Unkn	Amot 174 2 ug	26.95	89.00
Actin	Unkn	Amot 174 2 ug	25.91	89.00
Actin	Unkn	Amot 174 2 ug	25.02	89.00
Actin	Unkn	Amot 174 2 ug	24.40	89.00
GAPDH	Unkn	Amot 174 2 ug	22.26	84.50
GAPDH	Unkn	Amot 174 2 ug	22.32	84.50
GAPDH	Unkn	Amot 174 2 ug	22.38	84.50
GAPDH	Unkn	Amot 174 2 ug	22.25	84.50
Amot	Unkn	Amot 174 2 ug	25.70	83.50
Amot	Unkn	Amot 174 2 ug	25.78	83.50
Amot	Unkn	Amot 174 2 ug	26.01	83.50
Amot	Unkn	Amot 174 2 ug	25.82	83.50
PCNA	Unkn	Amot 174 2 ug	23.52	86.00
PCNA	Unkn	Amot 174 2 ug	23.65	86.00
PCNA	Unkn	Amot 174 2 ug	23.56	86.00
PCNA	Unkn	Amot 174 2 ug	23.50	86.00

Actin	Unkn	Amot 257 2 ug	22.65	89.50
Actin	Unkn	Amot 257 2 ug	22.77	89.50
Actin	Unkn	Amot 257 2 ug	22.92	89.50
Actin	Unkn	Amot 257 2 ug	22.95	89.50
GAPDH	Unkn	Amot 257 2 ug	21.35	85.00
GAPDH	Unkn	Amot 257 2 ug	21.44	85.00
GAPDH	Unkn	Amot 257 2 ug	21.49	85.00
GAPDH	Unkn	Amot 257 2 ug	21.40	85.00
Amot	Unkn	Amot 257 2 ug	25.09	83.50
Amot	Unkn	Amot 257 2 ug	25.16	84.00
Amot	Unkn	Amot 257 2 ug	25.14	83.50
Amot	Unkn	Amot 257 2 ug	25.10	83.50
PCNA	Unkn	Amot 257 2 ug	23.06	86.50
PCNA	Unkn	Amot 257 2 ug	23.14	86.50
PCNA	Unkn	Amot 257 2 ug	23.15	86.50
PCNA	Unkn	Amot 257 2 ug	23.07	86.50
Actin	NTC	N/A	N/A	None
Actin	NTC	N/A	38.40	None
Actin	NTC	N/A	N/A	None
Actin	NTC	N/A	N/A	None

mean Cq values for the Amot 174 samples were determined to be 25.57, 22.30, 25.83, and 23.56. For the Amot 257 samples, the mean Cq values were determined to be 22.82, 21.42, 25.12, and 23.10. Lastly, for the no-template control samples, the mean Cq was determined to be 38.40. The melt temperature analysis of this 2-µg revealed data similar to that which was collected in the preceding condition. For example, the melt temperature of each sample targeting ß-actin was approximately 89℃; the melt temperature of each sample targeting GAPDH was approximately 85 ℃; the melt temperature of each sample targeting Amot was approximately 84 ℃; and the melt temperature of each sample targeting PCNA was approximately 86 ℃ (Table 4). In the no-template control samples, on the other hand, no melt temperature could be determined. As mentioned earlier, the mean Cq values were essential to determining relative normalized expression. The Bio-Rad CFX Maestro software was used to generate the normalized expression graph for the 2-µg condition (Figure 5). From the normalized expression graph, it appeared that there was a significant decrease in Amot and PCNA expression in cells treated with the Amot 98 construct relative to those cells treated with the control construct. A similar trend was seen in those cells treated with the Amot 174 construct, as a significant decrease was seen in normalized expression for both Amot and PCNA. Lastly, it appeared that there was also a significant decrease in both Amot and PCNA expression in those cells treated with the Amot 257 construct.

Finally, qPCR was also performed for the 4-µg condition. Again, each cDNA sample from those cells treated with 4 µg of DNA was targeted for ß-actin, GAPDH, Amot, and PCNA. The Cq values of each target tested for in the different cDNA samples were determined using the CFX96 Real-Time System (Table 5). For the CNTRL samples, the mean Cq value for the ß-actin target was 19.48; the mean Cq value for the GAPDH target was 17.96; the mean Cq value for the Amot target was 22.83; and the mean Cq value for the PCNA target was 19.53. With the targets

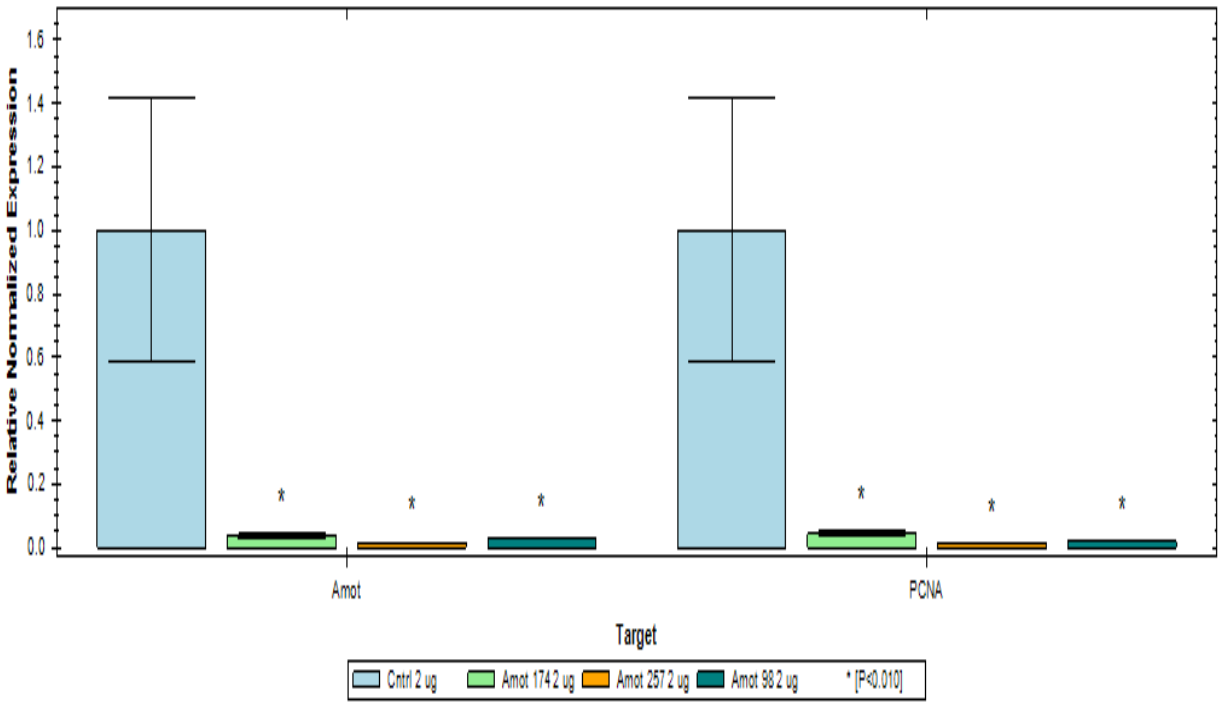

Figure 5: This figure depicts the normalized expression graph for the 2-µg condition. As mentioned, the expression within the control was set to 1.0. The error bars represent standard error of the mean. Significance was determined by a P-value less than 0.01.

Table 5: Cq values and melt temperatures across the 4-µg condition

Target	Content	Sample	Cq	Melt Temp. (℃)
Actin	Pos Ctrl	Cntrl 4ug	19.73	89.00
Actin	Pos Ctrl	Cntrl 4ug	19.44	89.00
Actin	Pos Ctrl	Cntrl 4ug	19.40	89.00
Actin	Pos Ctrl	Cntrl 4ug	19.35	89.00
GAPDH	Pos Ctrl	Cntrl 4ug	17.94	85.50
GAPDH	Pos Ctrl	Cntrl 4ug	17.98	85.00
GAPDH	Pos Ctrl	Cntrl 4ug	17.96	85.00
GAPDH	Pos Ctrl	Cntrl 4ug	17.95	85.00
Amot	Pos Ctrl	Cntrl 4ug	22.80	84.00
Amot	Pos Ctrl	Cntrl 4ug	22.87	84.00
Amot	Pos Ctrl	Cntrl 4ug	22.88	84.00
Amot	Pos Ctrl	Cntrl 4ug	22.77	84.00
PCNA	Pos Ctrl	Cntrl 4ug	19.54	86.50
PCNA	Pos Ctrl	Cntrl 4ug	19.44	86.50
PCNA	Pos Ctrl	Cntrl 4ug	19.49	86.50
PCNA	Pos Ctrl	Cntrl 4ug	19.63	86.50
Actin	Unkn	Amot 98 4ug	16.97	89.00
Actin	Unkn	Amot 98 4ug	16.58	89.00
Actin	Unkn	Amot 98 4ug	16.41	89.00
Actin	Unkn	Amot 98 4ug	16.29	89.00
GAPDH	Unkn	Amot 98 4ug	16.13	85.00
GAPDH	Unkn	Amot 98 4ug	16.03	85.00
GAPDH	Unkn	Amot 98 4ug	16.10	85.00
GAPDH	Unkn	Amot 98 4ug	16.07	85.00
Amot	Unkn	Amot 98 4ug	20.55	84.00
Amot	Unkn	Amot 98 4ug	20.40	84.00
Amot	Unkn	Amot 98 4ug	20.34	84.00
Amot	Unkn	Amot 98 4ug	20.41	84.00
PCNA	Unkn	Amot 98 4ug	17.77	86.50
PCNA	Unkn	Amot 98 4ug	17.64	86.50
PCNA	Unkn	Amot 98 4ug	17.61	86.50
PCNA	Unkn	Amot 98 4ug	17.75	86.50
Actin	Unkn	Amot 174 4ug	18.90	89.00
Actin	Unkn	Amot 174 4ug	18.89	89.00
Actin	Unkn	Amot 174 4ug	18.34	89.00
Actin	Unkn	Amot 174 4ug	18.57	89.00
GAPDH	Unkn	Amot 174 4ug	17.76	85.50
GAPDH	Unkn	Amot 174 4ug	17.79	85.50
GAPDH	Unkn	Amot 174 4ug	17.66	85.50
GAPDH	Unkn	Amot 174 4ug	17.67	85.00
Amot	Unkn	Amot 174 4ug	22.70	84.00
Amot	Unkn	Amot 174 4ug	22.68	84.00
Amot	Unkn	Amot 174 4ug	22.76	84.00
Amot	Unkn	Amot 174 4ug	22.76	84.00
PCNA	Unkn	Amot 174 4ug	19.72	86.50
PCNA	Unkn	Amot 174 4ug	19.97	86.50
PCNA	Unkn	Amot 174 4ug	20.02	86.50
PCNA	Unkn	Amot 174 4ug	-	None

Actin	Unkn	Amot 257 4ug	16.44	89.50
Actin	Unkn	Amot 257 4ug	16.48	89.50
Actin	Unkn	Amot 257 4ug	16.39	89.50
Actin	Unkn	Amot 257 4ug	16.37	89.50
GAPDH	Unkn	Amot 257 4ug	16.15	85.50
GAPDH	Unkn	Amot 257 4ug	16.18	85.50
GAPDH	Unkn	Amot 257 4ug	16.19	85.50
GAPDH	Unkn	Amot 257 4ug	16.15	85.50
Amot	Unkn	Amot 257 4ug	20.50	84.00
Amot	Unkn	Amot 257 4ug	20.62	84.00
Amot	Unkn	Amot 257 4ug	20.55	84.00
Amot	Unkn	Amot 257 4ug	20.52	84.00
PCNA	Unkn	Amot 257 4ug	17.66	86.50
PCNA	Unkn	Amot 257 4ug	17.70	86.50
PCNA	Unkn	Amot 257 4ug	17.72	86.50
PCNA	Unkn	Amot 257 4ug	17.88	86.50
Actin	NTC	N/A	N/A	None
Actin	NTC	N/A	N/A	None
Actin	NTC	N/A	N/A	None
Actin	NTC	N/A	N/A	None

listed in the same order, the mean Cq values of the Amot 98 samples were 16.56, 16.08, 20.43, and 17.69. The mean Cq values of the Amot 174 samples were 18.67, 17.72, 22.73, and 19.90. Lastly, the mean Cq values of the Amot 257 samples were 16.42, 16.17, 20.55, and 17.74. The no-template control samples did not yield any Cq values. In addition to the determination of the Cq values for each target, melt temperature analysis was performed to analyze the amplified targets (Table 5). Similar to the previous conditions, the melt temperature of each ß-actin gene product was approximately 89 ℃. Additionally, the melt temperature of each GAPDH gene product was approximately 85 ℃. Furthermore, the melt temperature of each Amot gene product was determined to be approximately 84 ℃. Finally, the melt temperature of each PCNA gene product was 86.5 ℃. In addition to failing to yield Cq values, the no-template control samples did not provide any melt temperature values. Through the use of the Bio-Rad CFX Maestro software once again, normalized expression of the two target genes was determined for each of the treatment constructs (Figure 6). In the cells treated with 4 µg of the Amot 174 construct, there appeared to be a significant decrease in the expression of Amot and in the expression of PCNA relative to those cells treated with 4 µg of the Scrambled CRISPR control plasmid. In the cells treated with 4 µg of the Amot 257 construct, there appeared to be a significant decrease in the expression of both Amot and PCNA relative to the control. Finally, in the cells treated with 4 µg of the Amot 98 construct, there was no significant decrease or increase in the expression of Amot. However, there was a significant decrease in the expression of PCNA in those cells treated with 4 µg of the Amot 98 construct.

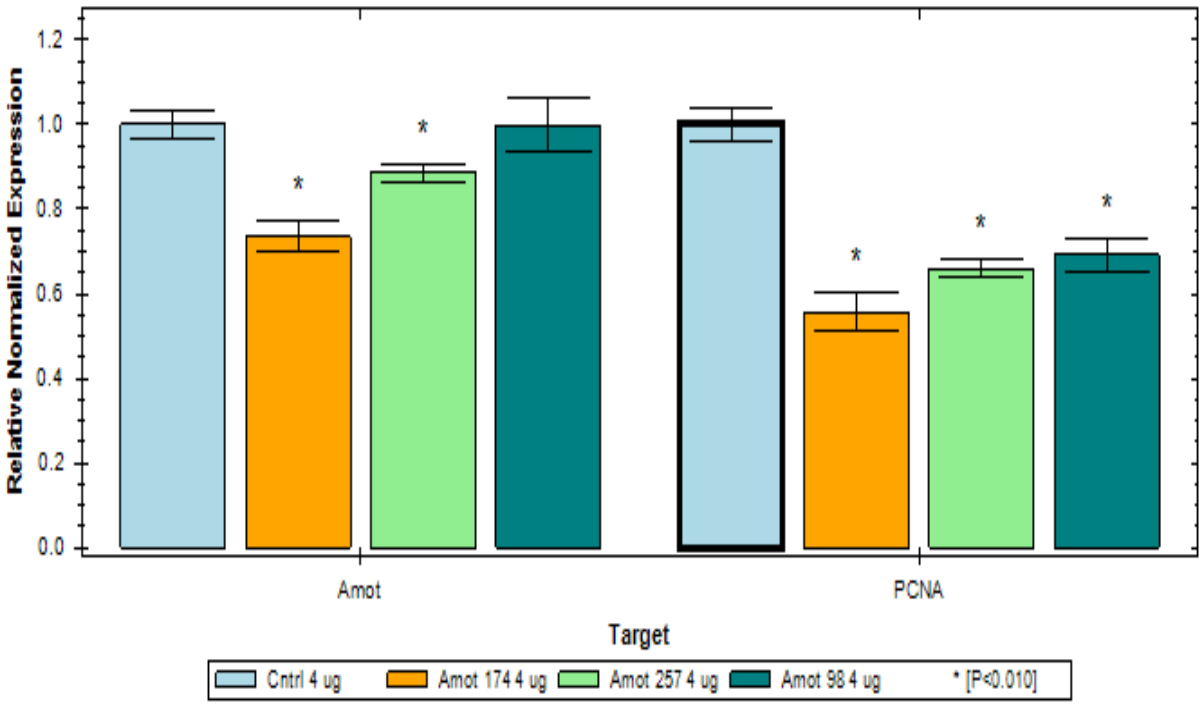

Figure 6: This figure depicts the normalized expression graph for the 4-µg condition. As mentioned, the expression within the control was set to 1.0. The error bars represent standard error of the mean. Significance was determined by a P-value less than 0.01.

Discussion

Although angiomotin was the main target of interest in the current study, proliferating cell nuclear antigen (PCNA) was also targeted in order to determine if there are any potential links between angiomotin expression and PCNA expression. Proliferating cell nuclear antigen is considered to be a member of the DNA sliding clamp family of proteins, along with the ß-subunit of DNA polymerase III from *E. coli*. PCNA is considered to be a member of this protein family due to the fact that PCNA can form a ring around DNA (Strzalka and Ziemienowicz 2011). PCNA is known to have a role in the processes of DNA replication, DNA repair, cell cycle regulation, and even apoptosis. These roles were elucidated when it was discovered that PCNA can bind DNA, cyclins, DNA polymerase, cyclin-dependent kinases, and even p21 (Maga and Hübscher 2003). While it is known that PCNA can interact with all of the aforementioned proteins, it is not yet known how the exact impact of PCNA is regulated. One study believes that the varying binding affinities between PCNA and its substrates may contribute to its regulation, so these binding affinities should be determined to elucidate the mechanisms of PCNA within the cell (Maga and Hübscher 2003). The connection of PCNA to processes essential to the cell's survival, such as DNA replication and repair, is the primary reason why PCNA expression is examined alongside Amot expression. In reducing the expression of Amot in tumorigenic cells, it is hoped that these cells would show decreased cell viability. Therefore, if PCNA shows a similar decrease in expression when Amot is silenced, then it is possible that Amot may be an effective target for the treatment of cancer cells. This potential association is kept in mind throughout the analysis of the data of the current study.

The raw data of the 1-µg condition reveals that this part of the experiment was performed with success. For example, for each target, the Cq values are all very precise. This precision indicates that the pipetting of both the primer mixes and the cDNA samples into the 96-well plate was performed effectively, avoiding the addition of different amounts of either the sample or the primer mix to each well. Furthermore, the melt temperature analysis reveals that contamination was avoided. There are two types of contamination: primer-dimers and genomic contamination. Primer-dimers occur when the primers base-pair, and they are usually detected by a melt temperature of about 77 ℃. Genomic contamination, on the other hand, occurs when contamination is introduced from the external environment, and it is characterized by a melt temperature of about 95 ℃. With the melt temperatures for each target being between 84 ℃ and 89 ℃, it is apparent that there was no contamination in the samples. Furthermore, the lack of Cq values and melt temperatures for the no-template controls strengthen the overall accuracy of this condition, for these are the results expected of no-template controls. Given both the accuracy and precision of the experiment, the data can certainly be useful for making conclusions. Earlier it was indicated that there was a significant decrease in Amot and PCNA expression in the cells treated with 1 µg of the Amot 174 CRISPR construct. In those cells treated with 1 µg of Amot 257, however, the expression of Amot increased significantly, while the expression of PCNA had no significant increase or decrease. Likewise, in those cells treated with 1 µg of the Amot 98 construct, there was a significant increase in Amot and in PCNA. It is strange that only the Amot 174 construct was successful at this concentration, but this difference could be caused by a few factors. First, it is possible that the Amot 174 construct is simply more effective at silencing Amot than the other two constructs. Additionally, the “increase” in Amot expression seemingly caused by the Amot 98 and the Amot 257 constructs could have been due to the fact that the

successfully edited cells died prior to RNA extraction. If Amot and PCNA expression are truly intimately coordinated, then a decrease in Amot would likely correspond to a decrease in cell viability and survivability. If the cells died before the RNA extraction, then the cells that were present were likely those that were not successfully edited by the Amot 98 and Amot 257 constructs. This possibility must be considered prior to ruling out the other constructs for subsequent studies.

Examination of the 2-µg data reveals a part of the experiment that may not have been performed with the same level of precision and accuracy as the previous condition. In each quantitative polymerase chain reaction that was performed, two different housekeeping genes were targeted to determine the fidelity of the whole reaction. Housekeeping genes, such as those that code for ß-actin and GAPDH, are considered to be constitutively active; therefore, the Cq values for these targets should always be relatively low. However, when targeting ß-actin in the 2 µg CNTRL samples, the Cq values were either above 30 or unavailable. Cq values above 30 indicate that the target was present in very low quantities to begin with or that there was really no target present at all. The high Cq values for ß-actin in the 2 µg CNTRL samples could have been due to a lack of effectively mixing the primer mix, or they could have been caused by improper pipetting. Regardless, the abnormal Cq values for this housekeeping gene could certainly skew the normalized expression, for normalized expression is determined by comparing the expression of the target gene to the expression of a positive control. Therefore, conclusions drawn from the 2-µg condition may not be as useful as those drawn from conditions that were performed with greater experimental precision and accuracy. Despite the abnormal Cq values for ß-actin in the CNTRL 2 µg samples, there was no contamination present throughout this condition: there were no melt temperatures corresponding to either genomic contamination or primer-dimers.

According to the normalized expression graph from the 2-µg condition (Figure 5), there was a significant decrease in the expression of Amot and PCNA in cells transfected with the Amot 98 construct, the Amot 174 construct, and the Amot 257 construct. However, as mentioned above, these results should not be used to draw meaningful conclusions. Instead, repetition of this particular condition would be needed to verify if each construct really caused significant decreases in Amot expression and PCNA expression.

Analysis of the data generated from the 4-µg condition reveals another part of the experiment that was performed with precision and accuracy. For example, for each set of targets, the Cq values were all very close. As in the 1-µg condition, this precision demonstrates effective pipetting throughout the preparation of the 96-well plate. In addition, the Cq values of the housekeeping genes are predictably low, demonstrating that the positive control samples could be used as an effective source of comparison for the expression of Amot and PCNA. Furthermore, the melt temperature analysis conveys a lack of contamination: there are no melt temperatures indicative of either primer-dimers or genomic contamination. Finally, the lack of Cq values and melt temperatures from the no-template control samples emphasizes the avoidance of contamination in this condition. From the normalized expression graph (Figure 6), it is apparent that there was a significant decrease in expression of both Amot and PCNA in those cells treated with either 4 µg of the Amot 174 construct or 4 µg of the Amot 257 construct. However, the cells treated with the Amot 98 construct demonstrated a significant decrease in PCNA expression and no significant change in Amot expression. Similar to the evaluation of the 1-µg condition, it is possible that the lack of reduction in Amot expression seen in cells treated with 4 µg of Amot 98 could be due to the fact that the edited cells died. Conversely, it is also possible that the Amot 98 construct is simply not as effective as the other constructs at silencing

Amot. Again, repetition is essential to determine the efficacy of each construct. For example, even though treatment with 4 µg of Amot 174 or 4 µg Amot 257 seems to effectively attenuate Amot expression and cause a concurrent decrease in PCNA expression, repetition is still needed to verify the association between Amot and PCNA and to determine if these constructs are truly effective at this concentration.

Overall, it does appear that there is an association between Amot expression and PCNA expression. For example, reductions in Amot expression were typically mirrored by reductions in PCNA expression, while increases in Amot expression were also mirrored by increases in PCNA expression. However, it is important to remember that this association does not necessarily indicate a direct connection between the two targets. It was mentioned that PCNA binds to many proteins involved in many different cellular processes, so it is likely that the association is a complicated one. Furthermore, the current study revealed that the Amot 174 CRISPR construct was the most effective construct at silencing Amot across all conditions. Though the results of the 2-µg condition are not entirely conclusive, the 1-µg and 4-µg conditions reveal a significant decrease in both Amot expression and PCNA expression in those cells treated with the Amot 174 construct. Therefore, future studies should include repetition of the current study in order to verify the efficacy of the Amot 174 construct at reducing Amot expression and to determine which concentration of this construct is most effective. End-point PCR could be performed in conjunction with qPCR in order to confirm that angiomotin was successfully edited. Furthermore, western blots should be performed to determine if the significant reductions in Amot and PCNA gene expression can be confirmed by significant reductions in the protein levels within treated cells. Additionally, given the association between Amot and PCNA expression, cell viability assays should be performed to examine if silencing Amot significantly

reduces cell viability. Lastly, to biochemically verify reduction in cell viability, it would be worthwhile to target expression of various pro-apoptotic factors alongside Amot and PCNA expression. Given the numerous connections between angiomotin and breast cancer pathology, further studies are certainly justified to elucidate whether or not angiomotin could serve as an effective target for breast cancer treatment.

Acknowledgements

I would first and foremost like to thank my heavenly Father for all of the opportunities He has blessed me with. I would also like to recognize Dr. William Ranahan, who has been a stellar professor, advisor, mentor, and friend to me throughout my four years at this school. I am beyond thankful for his patience throughout the research process and his willingness to continue to work with me in the lab despite all of the chaos of this abbreviated semester. I also want to thank all of the faculty of the Biology & Chemistry Department for their crucial roles in developing me into the student and person I am today. I have truly enjoyed my time at Oral Roberts, and I will genuinely miss all of the faculty. I am also beyond grateful for my parents, who have been such an amazing support group for me throughout my life. Finally, I would like to thank my lovely wife, Ciera Andrews, for her patience with me as I spent countless evenings in the lab.

Literature Cited

Adler JJ, Heller BL, Bringman LR, Ranahan WP, Cocklin RR, Goebl MG, Oh M, Lim HS, Ingham RJ, Wells CD. 2013. Amot130 adapts atrophin-1 interacting protein 4 to inhibit yes-associated protein signaling and cell growth. J Biol Chem. 288(21):15181–15193. doi:10.1074/jbc.M112.446534.

Adler JJ, Johnson DE, Heller BL, Bringman LR, Ranahan WP, Conwell MD, Sun Y, Hudmon A, Wells CD. 2013. Serum deprivation inhibits the transcriptional co-activator YAP and cell growth via phosphorylation of the 130-kDa isoform of Angiomotin by the LATS1/2 protein kinases. Proc Natl Acad Sci U S A. 110(43):17368–17373. doi:10.1073/pnas.1308236110.

BreastCancer.org. 2018. Types of Breast Cancer: Non-Invasive, Invasive and More. https://www.breastcancer.org/symptoms/types.

DeSantis C, Ma J, Bryan L, Jemal A. 2014. Breast cancer statistics, 2013. CA Cancer J Clin. 64(1):52–62. doi:10.3322/caac.21203. [accessed 2020 Oct 10]. https://acsjournals.onlinelibrary.wiley.com/doi/full/10.3322/caac.21203.

Ernkvist M, Birot O, Sinha I, Veitonmaki N, Nyström S, Aase K, Holmgren L. 2008. Differential roles of p80- and p130-angiomotin in the switch between migration and stabilization of endothelial cells. Biochim Biophys Acta - Mol Cell Res. 1783(3):429–437. doi:10.1016/j.bbamcr.2007.11.018.

Jemal A, Bray F, Ferlay J. 1999. Global Cancer Statistics: 2011. CA Cancer J Clin. 49(2):1,33-64. doi:10.3322/caac.20107.Available. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10200776.

Jiang WG, Watkins G, Douglas-Jones A, Holmgren L, Mansel RE. 2006. Angiomotin and angiomotin like proteins, their expression and correlation with angiogenesis and clinical outcome in human breast cancer. BMC Cancer. 6:1–11. doi:10.1186/1471-2407-6-16.

Klug WS, Cummings MR, Spencer CA, Palladino MA. 2015. Concepts of Genetics. 11th ed. Boston: Pearson.

Maga G, Hübscher U. 2003. Proliferating cell nuclear antigen (PCNA): A dancer with many partners. J Cell Sci. 116(15):3051–3060. doi:10.1242/jcs.00653.

Maughan KL, Lutterbie MA, Ham PS. 2010. Treatment of Breast Cancer. [accessed 2020 Oct 11]. www.aafp.org/afpAmericanFamilyPhysician1339.

Overview of the Breast - Breast Pathology | Johns Hopkins Pathology. 2020. [accessed 2020 Oct 12]. https://pathology.jhu.edu/breast/overview.

Pennisi E. 2013. The CRISPR Craze. Science (80-). 341(6148).

Ranahan WP, Han Z, Smith-Kinnaman W, Nabinger SC, Heller B, Herbert BS, Chan R, Wells CD. 2011. The adaptor protein AMOT promotes the proliferation of mammary epithelial cells via the prolonged activation of the extracellular signal-regulated kinases. Cancer Res. 71(6):2203–2211. doi:10.1158/0008-5472.CAN-10-1995. [accessed 2020 Oct 10]. http://cancerres.aacrjournals.org/.

Strzalka W, Ziemienowicz A. 2011. Proliferating cell nuclear antigen (PCNA): A key factor in DNA replication and cell cycle regulation. Ann Bot. 107(7):1127–1140. doi:10.1093/aob/mcq243. [accessed 2020 Nov 5]. www.aob.oxfordjournals.org.

Xiao-Jie L, Hui-Ying X, Zun-Ping K, Jin-Lian C, Li-Juan J. 2015. CRISPR-Cas9: A new and promising player in gene therapy. J Med Genet. 52(5):289–296. doi:10.1136/jmedgenet-2014-102968.