THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DIVISION OF SCIENCE

DETERMINING THE ROLE OF THE RNA BINDING PROTEIN HUR IN THE RNA STABILITY OF ANTIZYME AND ANTIZYME INHIBITOR

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SPRING 2016

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biochemistry and Molecular Biology
with honors in Biochemistry and Molecular Biology

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ABSTRACT

Non-melanoma skin cancer (NMSC) is the most commonly diagnosed cancer in the United States. NMSC has two common types: basal cell carcinoma and squamous cell carcinoma, which can be caused by exposure to UV radiation. The polyamine pathway, particularly the upregulation of ornithine decarboxylase (ODC), has long been studied and correlated with tumorigenesis, especially in skin. Two endogenous regulators of ODC, antizyme (AZ) and antizyme inhibitor (AZI), have been implicated in tumorigenesis as well, although via poorly understood mechanisms. AZ binds to ODC and regulates ODC's degradation by the 26S proteasome. AZI is able to bind to AZ to thus prevent ODC degradation. Previously, it was found that ODC was regulated by the RNA binding protein, Human Antigen R (HuR). In our current studies we plan to test whether AZ and AZI are also regulated by HuR in the NMSC mouse cell line model.

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LIST OF ABBREVIATIONS

3’UTR	3’ untranslated region
5’UTR	5’ untranslated region
APC	adenomatous polyposis coli
ARE	adenosine- and uracil-rich element
AZ	antizyme
AZBE	antizyme 1 binding element
AZI	antizyme inhibitor
BCC	basal cell carcinoma
DFMO	α-difluoromethylornithine
HuR	human antigen R
mRNA	messenger RNA
NMSC	non-melanoma skin cancer
ODC	ornithine decarboxylase
ORF	open reading frame
PLP	pyridoxal 5’-phosphate
RRM	RNA recognition motif
SCC	squamous cell carcinoma
UV	ultraviolet

ACKNOWLEDGEMENTS

Thank you to Dr. Shannon Nowotarski for being an excellent research advisor, for allowing me to contribute to an amazing project, and for guiding me on how to write my thesis. You have pushed me to be the best I can be, and for that I cannot thank you enough. Thank you to Anh Mai, Sean Flannery, and Torey Jordan for helping me complete the laboratory work.

Thank you to Dr. Tami Mysliwiec and Leslie Turner for your assistance in the laboratory and using the imager. Also, thank you to Dr. Bill Zachowicz for your guidance and endless amount of support. Thank you to my father, Gary Neiman, for providing reassurance and encouragement during research and writing my thesis. Lastly, thank you to my brother, Andrew Neiman, for taking the time and reading my final draft. I would not have been able to do this without the assistance of all of you.

Chapter 1

Introduction

1.1 Non-melanoma skin cancer

Non-melanoma skin cancer (NMSC) is the most commonly diagnosed cancer in the United States. NMSC is categorized into two common types, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Over two million people are diagnosed with NMSC each year, with some having multiple diagnoses per clinical visit (Kim and Armstrong, 2012). Like all cancers, these tumors are caused by genetic mutations within the cell. The primary carcinogen for NMSC is ultraviolet (UV) radiation (Ridky, 2007).

UV radiation has three forms UVA (315-400nm wavelength), UVB (280-315nm wavelength), and UVC radiation (200-280nm wavelength) (Dupont et al., 2013). UVB radiation is the wavelength of radiation that can cause DNA damage directly. UVA can also cause DNA damage but more indirectly by the free radicals it produces (Schmidt, 2012). UVC is the most powerful form of UV radiation; however, it is filtered by the ozone (Dupont et al., 2013). Both UVA and UVB can contribute to the development of NMSC. Cyclobutane pyrimidine dimers and 6-4 photoproducts can form due to DNA exposure to these forms of UV radiation (Dupont et al., 2013). Usually these genetic mutations can be repaired through repair mechanisms such as nucleotide excision repair. However, it is when these mutations are not repaired that NMSC can arise (Dupont et al., 2012).

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) occur in keratinocytes located in the epidermis region of the skin (Figure 1.1). BCC is the most common form of NMSC, while SCC is the second most common with a 4:1 ratio of cases respectively (Ridky,

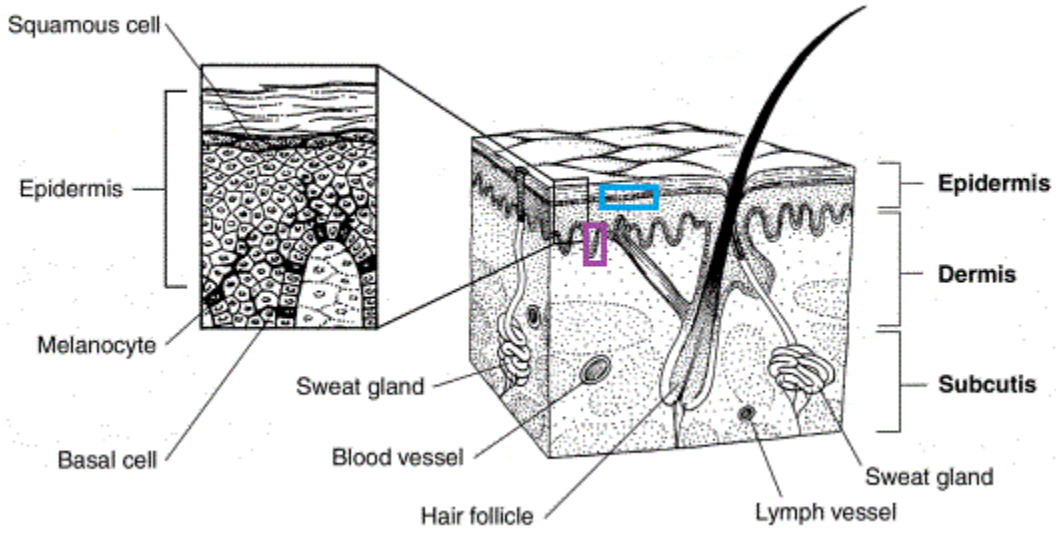

Figure 1.1 Layers of the skin and cell location

The layers of the skin are the epidermis, dermis, and subcutis, with the epidermis being the outermost layer of the skin. Basal cells are shown in purple near the bottom of the epidermis, and squamous cells shown in blue are located near the top of the epidermis. The basal cells and squamous cells can be mutated and lead to the formation of two types of NMSC, BCC and SCC respectively.

Image source: Skin Cancer: Basal and Squamous Cell. 2015. American Cancer Society. [cited 2015 Nov 27] Available from: http://www.cancer.org/cancer/skincancer-basalandsquamouscell/detailedguide/skin-cancer-basal-and-squamous-cell-what-is-basal-and-squamous-cell

2007) (Dubas and Ingraffea, 2013). Statistics show that 3.5 million cases of NMSC are diagnosed every year and out of these cases, 80% are BCCs. Interestingly, NMSC is more common than lethal melanoma skin cancer which accounted for 73,000 cases in 2015 (Figure 1.2) (Skin Cancer Facts, 2015). NMSC is rarely lethal accounting for about 2,000 fatalities each year. Alarmingly, the number of diagnoses of NMSC is increasing every year. Treatment for NMSC usually involves surgical removal of the cancerous growth, followed by treatment with chemotherapy or radiation to remove any remaining cancerous cells (Skin Cancer: Basal and Squamous Cell, 2015).

1.2 The polyamine pathway

Polyamines are cations that can bind to negatively charged molecules in order to stabilize them. Polyamines are capable of modifying ion transport channels as well as controlling translation (Nilsson et al., 2005). DNA structure and signaling pathways can be altered by polyamines which implicate polyamines in regulating gene expression. Researchers have found that the levels of polyamines can alter the structure of DNA from right-handed to left-handed. Thus, the conformational changes due to polyamine binding can cause DNA to open and be actively transcribed. Polyamines can also aid in DNA-protein interactions by enhancing gene-regulatory protein binding (Thomas and Thomas, 2003). Moreover, it was found that when there are lower levels of polyamines, MAP kinase can become active in order to activate ornithine decarboxylase (ODC). This activation of ODC can lead to a higher production of polyamines and tumorigenesis.

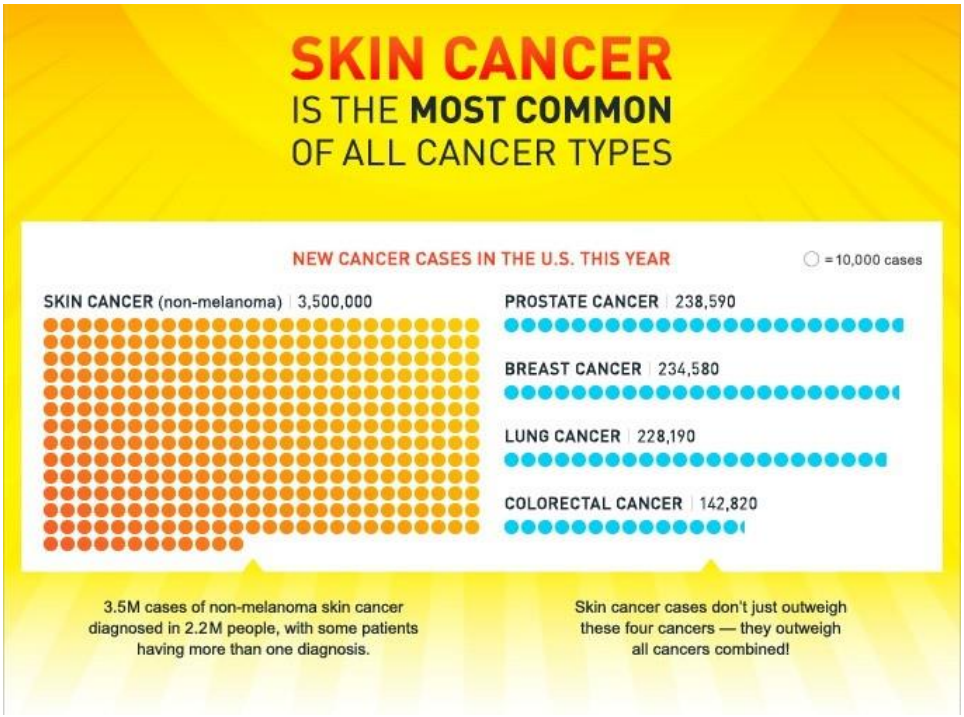

Figure 1.2 Cases of NMSC in a year

In one year there were 3,500,000 diagnosed cases of NMSC. This amount largely outweighs the amount of cases for any other type of cancer, thus illustrating that NMSC is the most commonly diagnosed form of cancer. As shown, NMSC is more common than prostate cancer, breast cancer, lung cancer, and colorectal cancer combined.

Image source: Don't Fry: Preventing Skin Cancer. 2015. American Cancer Society. [cited 2016 Feb 1] Available from: http://www.cancer.org/research/infographicgallery/skin-cancer-prevention?gclid=CPXc5YWFwboCFVOf4AodulEARQ

The polyamines, putrescine, spermidine, and spermine, are formed via a tightly regulated mechanism (Figure 1.3). In the pathway, ornithine is formed from L-methionine and L-arginine as an output of the urea cycle (Pegg et al., 2003). Ornithine is decarboxylated and converted to putrescine by ODC. Putrescine is converted to spermidine by the addition of an aminopropyl group by spermidine synthase. Spermidine can be converted to spermine with the addition of another aminopropyl group by spermine synthase. Antizyme (AZ) has been found to endogenously regulate ODC by binding to the ODC monomer and relocating ODC for degradation to the 26S proteasome. Antizyme inhibitor (AZI) has been shown to bind to AZ, thus preventing ODC degradation (Gilmour, 2007).

1.2.1 Polyamine pathway and cancer

In the 1960s it was discovered that there was a correlation between dysregulated polyamine levels, primarily via the upregulation of ODC, and cancer. Researchers showed that there were high levels of polyamines in the urine of patients with cancer (Gerner and Meyskens, 2004). Ahmad et al. showed that UV light could affect ODC levels by exposing mice that overexpress ODC to UVB radiation. When exposed to UVB radiation, epidermal tumors formed and ODC activity was found to increase. The effects of the ODC pharmacological inhibitor, DFMO, on tumor formation was then tested on mice exposed to UV radiation. Mice were given DFMO in their drinking water, and studied for 30 weeks. None of the DFMO-treated mice developed epidermal tumors; however, the control mice developed tumors suggesting that ODC expression plays a causative role in skin tumor development (Ahmad et al., 2001).

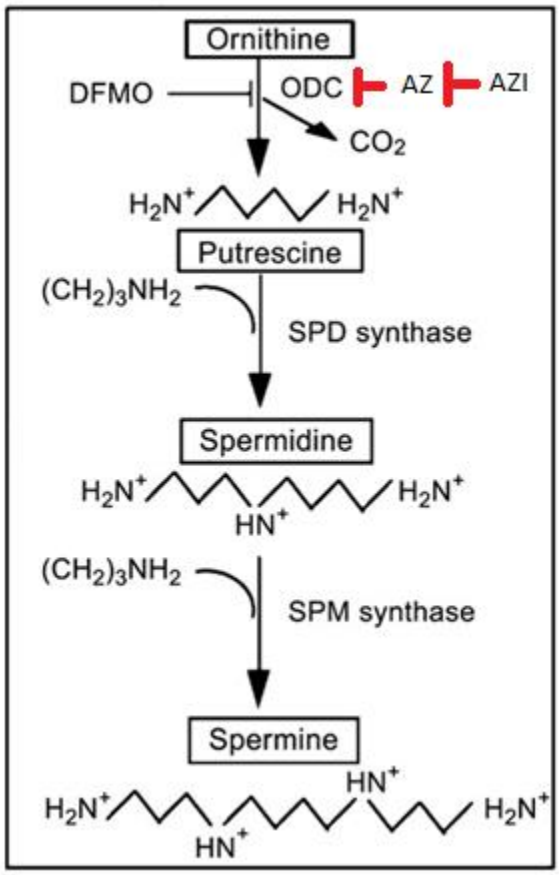

Figure 1.3 The polyamine pathway

Ornithine is decarboxylated by ODC to become putrescine. An aminopropyl group is added to putrescine by spermidine synthase (SPD synthase) to make spermidine. Another aminopropyl group is added to spermidine by spermine synthase (SPM synthase) to form spermine. AZ is an inhibitor of ODC, and AZI is the inhibitor of AZ. AZ can form a complex with ODC in order to cause ODC degradation, and AZI can bind to AZ to prevent binding and inhibition of ODC (Gilmour, 2007).

Image source: Modified from Lisa M. Shantz (Penn State College of Medicine)

Moreover, in colon cancer ODC has been shown to be upregulated. Researchers found that adenomatous polyposis coli (APC) is mutated in a form of colon cancer. This mutation causes MYC to be upregulated and MYC in turn upregulates ODC by increasing the rate of transcription of *odc*. Interestingly, the mutation of APC can also affect the inhibitor of ODC, AZ. AZ activity is decreased when APC is mutated which leads to the increased activity of ODC. In addition to these examples, the polyamine pathway has been implicated in development of tumors in the breast, lung, and prostate further attesting to the importance of this pathway in tumorigenesis (Gerner and Meyskens, 2004).

1.3 ODC

As stated above, ODC is the first metabolic enzyme in the polyamine pathway and converts the amino acid ornithine to the diamine putrescine. ODC is tightly regulated via transcription and translation. In previous research it was suggested that the 3' untranslated region (UTR) of ODC can decrease the inhibitory effect of the 5'UTR. It was shown that this cooperation between the UTRs of ODC could be due to factors within the cell during the cell cycle (Lorenzini and Scheffler, 1997). These data suggest that ODC is highly regulated by its UTRs post-transcriptionally.

ODC can also be regulated at the level of protein degradation by AZ. AZ prevents the dimerization of ODC thereby rendering ODC inactive (Perez-Leal and Merali, 2012).

1.4 AZ and AZI

AZ is an endogenous inhibitor of ODC and, as its name suggest, has anti-enzyme activity. It was shown that ODC and AZ form a complex. When the AZ-ODC complex is formed, ODC activity decreases and ODC can be degraded. In fact, AZ promotes the degradation of ODC through the 26S proteasome when the AZ-ODC complex is formed. The AZ-ODC complex can also dissociate in order to activate ODC (Coffino, 2001).

AZ levels depend on the polyamine content of the cell. Specifically, AZ is produced via translational frameshifting. Without the frameshift, translation would stop at the UGA stop codon of the first open reading frame (ORF). When polyamine levels are high, a frameshift occurs, which allows translation to continue until the end of the second ORF of AZ (Figure 1.4). This frameshift is needed for the production of a functional protein. The exact mechanism of this phenomenon is currently unknown (Kahana, 2009).

There are multiple forms of AZ. AZ-1 is the isoform that has a high affinity for and therefore primarily acts on ODC. When AZ-1 binds to ODC, ODC undergoes a conformational change that exposes its C-terminus for ubiquitin-independent degradation. The 26S proteasome recognizes the C-terminal of ODC for degradation in a process that does not cause the degradation of AZ. Specifically, the C-terminus of AZ is what allows AZ to bind to ODC, and the N-terminus helps promote ODC degradation by the proteasome (Kahana, 2009).

AZI is an endogenous inhibitor to all forms of AZ, and it also plays a role in regulating cell growth (Kahana, 2009). AZI has a higher affinity for AZ, higher than the affinity AZ has for ODC (Figure 1.5) (Mangold, 2006). AZI was first discovered in rat liver. Upon its discovery, it was thought that AZI could be a variant of ODC since both proteins are similar in size and composition. However, it was shown that between ODC and AZI, AZI has no decarboxylase

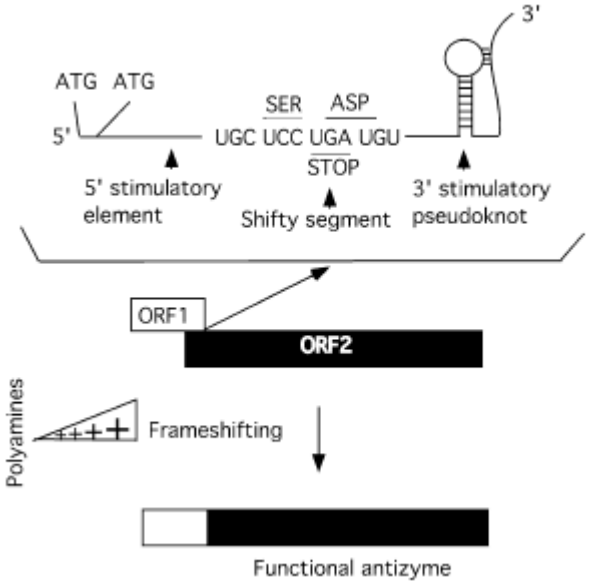

Figure 1.4 AZ translational frameshift

The first open reading frame is shown at the top. Without the frameshift, translation would stop at the UGA stop codon. Polyamines cause the frameshift event and allow for translation of AZ to continue until the end of the second open reading frame making a functional protein. The mechanism behind this phenomenon is currently unknown.

Image source: Kahana C. 2009. Antizyme and antizyme inhibitor, a regulatory tango. Cellular and Molecular Life Sciences. 66(15): 2479-2488.

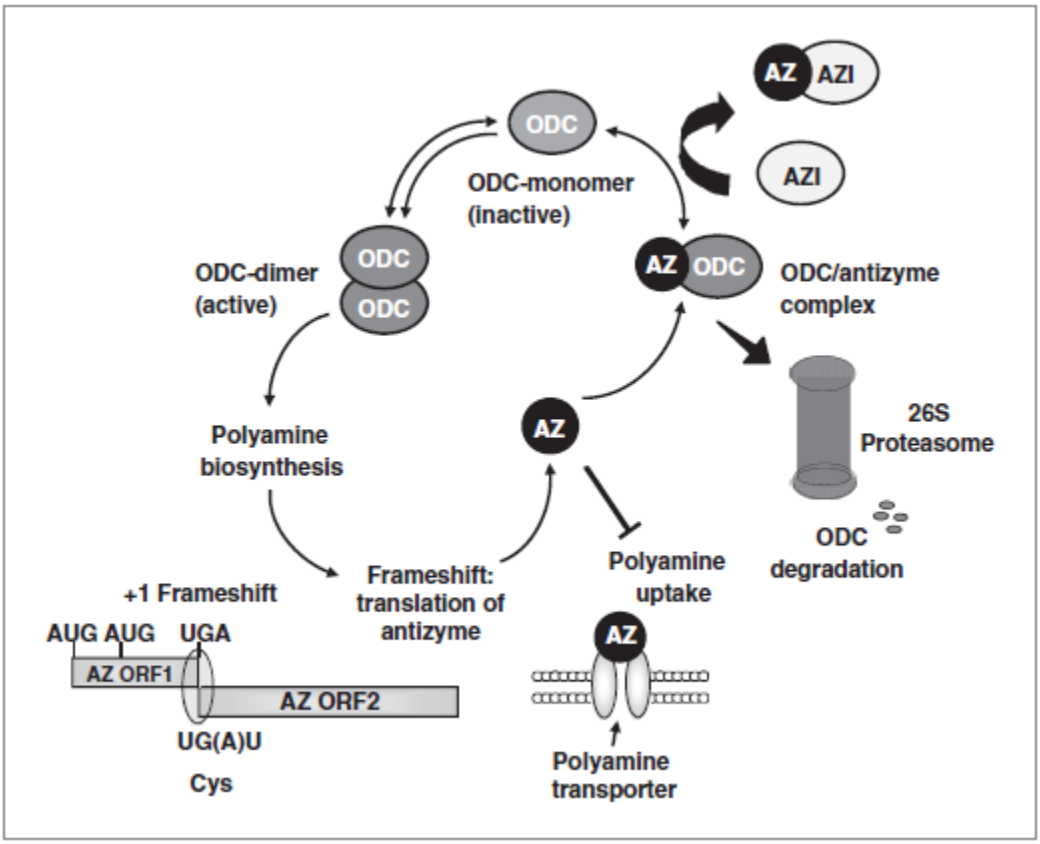

Figure 1.5 Interactions of polyamines, ODC, AZ, and AZI

The homodimer of ODC increases polyamine synthesis, which leads to the translational frameshift of AZ. AZ prevents polyamine increases by binding to ODC to promote ODC degradation by the 26S proteasome. AZI can bind to AZ in order to release ODC in its monomeric form (Olsen and Zetter, 2011).

Image source: Olsen RR, Zetter BR. 2011. Evidence of a Role for Antizyme and Antizyme Inhibitor as Regulators of Human Cancer. Molecular Cancer Research. 9(10): 1285-1293.

activity and lacks sequences to the active site for the cofactor pyridoxal 5'-phosphate (PLP), which ODC relies on (Mangold, 2006). Moreover, AZI lacks the region in the C-terminus that AZ uses to help promote degradation of ODC by the 26S proteasome (Mangold, 2006). For mouse ODC there is an AZ 1 binding element (AZBE) that resides between residues 117-140. This binding element is only accessible in the monomeric form of ODC. It is believed that AZI also has the AZBE within these same residues. Both AZI and ODC have dimeric forms that are extremely similar (Figure 1.6). AZI in the monomeric form has two domains, an α/β barrel domain and a domain similar to a Greek key β-sheet domain. The α/β barrel domain contains eight parallel strands, and the β-sheet domain contains two sheets. Thus, AZI and ODC have similar structural properties (Albeck et al., 2008).

1.5 RNA binding proteins

1.5.1 5' and 3' untranslated region (UTR) and mRNA stability

Messenger RNA (mRNA) is formed after transcription of DNA and is subsequently translated to form proteins. A mature mRNA contains both a 5' cap and a 3' poly(A) tail which are important for mRNA stability and translation.

Both, the 5'UTR and the 3'UTR can signal for mRNA to be degraded. The 5'UTR can also be important for the stabilization of mRNA. The 3'UTR can regulate the decay of the mRNA (Brennan and Steitz, 2001). Numerous proteins are capable of binding to certain 3'UTRs in order to stabilize the mRNA and prevent it from decay, possibly by protecting the poly(A) tail (Brennan and Steitz, 2001). One such example is α-globin which can be regulated by its 3'UTR

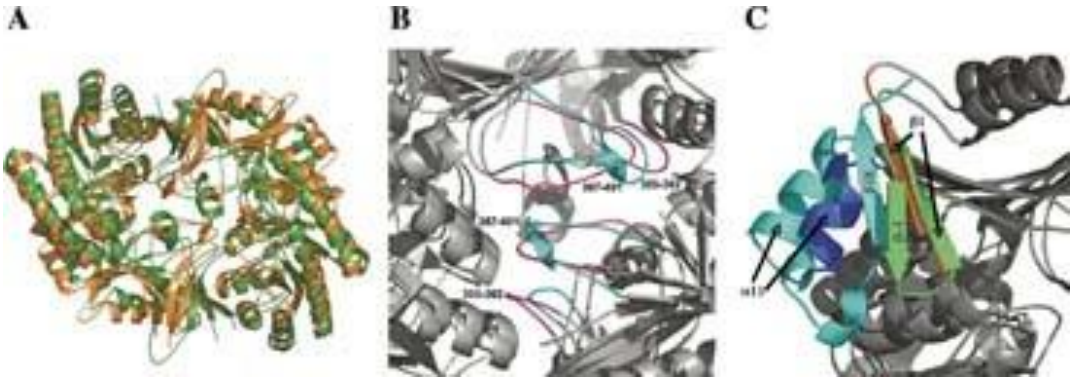

Figure 1.6 Comparison of AZI and ODC structures

(A) The dimeric form of AZI-1 (orange) and ODC (green) show the structural similarities. (B) The variable loops that connect the monomers to form the dimer shows different conformational changes between the two proteins. Teal represents AZI, and magenta represents ODC. (C) The N and C terminals of ODC and AZI. The N terminals in ODC are in green, and the C terminals are in blue. The N terminals in AZI are in red, and the C terminals are in cyan. AZI has an extra segment in the C terminal compared to ODC. The lack of segment in the C terminal of ODC may aid in ODC's interaction with AZ (Albeck et al., 2008).

Image source: Albeck S, et al. 2008. Crystallographic and biochemical studies revealing the structural basis for antizyme inhibitor function. Protein Science. 17(5): 793-802.

through a messenger ribonucleoprotein complex that binds to it and stabilizes the mRNA (Wang et al., 1995).

Within the 3'UTR there are adenosine- and uracil-rich elements (AREs) present which can influence the degradation of the mRNA. AREs can destabilize or stabilize a given mRNA depending on the cellular conditions and if an RNA binding protein (RBP) binds to the ARE. Various proteins can bind to an ARE to confer stability. In fact, 14 different proteins have been found that are capable of binding to these AU rich regions with some being able to affect mRNA stability (Brennan and Steitz, 2001).

RBPs are proteins that bind to mRNA at RNA recognition motifs (RRMs). These proteins provide protection for the mRNA in order to prevent degradation. One family of RBPs are Hu proteins which regulate mRNA stability (Hinman and Lou, 2008).

1.5.2 Human Antigen R RNA binding protein

HuR is a member of the Hu protein family. HuR is a protein that can respond to cellular stress by enhancing the expression of some anti-apoptotic proteins. HuR can bind to certain AREs within the 3'UTR in order to regulate stability of a given mRNA (Hinman and Lou, 2008).

Originally, HuR was discovered due to its importance in proper neuronal development. Subsequently, the protein was discovered to have ARE binding capabilities. When HuR is over expressed, mRNA is stabilized with class I, one to three copies of AUUUA in the U-rich regions, and class II AREs, at least two overlapping copies of UUAUUUA(U/A)(U/A). It is thought that HuR stabilizes the mRNA by protecting the body of the mRNA, and possibly by sequestering

factors necessary for degradation. When HuR is knocked out, mRNAs that contain AREs are degraded (Brennan and Steitz, 2001).

HuR contains three RRMs. Removal of the third RRM causes HuR to be unable to stabilize mRNA that contain AREs (Brennan and Steitz, 2001). Between the second and third RRM is a shuttling sequence called HuR nucleocytoplasmic shuttling sequence (HNS) which allows the protein to shuttle from the nucleus to the cytoplasm (Fan and Steitz, 1998). Crosslinking experiments have shown that HuR can bind to mRNAs in the nucleus as well as the cytoplasm. Researchers have found that when cells are damaged by UV radiation, HuR localizes to the cytoplasm; often this will lead to the stabilization of the HuR binding mRNAs (Brennan and Steitz, 2001).

Moreover, it has been shown that polyamines can influence HuR's ability to bind to c-Myc mRNA via the phosphorylation of chk2. When polyamine levels are low, chk2 is inactive causing HuR to not be phosphorylated. Without phosphorylated HuR, the affinity of HuR for the c-Myc mRNA is decreased. Upon the addition of putrescine, a downstream effecter of ODC, chk2 becomes active and phosphorylates HuR (Liu et al., 2009).

HuR has also been linked to cancer. Researchers isolated cancerous human liver cells to study the 3'UTR of C/EBPβ mRNA. The 3'UTR of the mRNA contains an ARE in which HuR can bind to stabilize the mRNA. When the ARE region of the 3'UTR for C/EBPβ was deleted, tumor suppression activity was lowered. Researchers found that HuR bound to the ARE in order to prevent tumor suppression (Sun et al., 2012).

In 2010, it was discovered that HuR was able to bind to the 3'UTR of ODC in mouse spindle carcinoma cells. The binding of HuR to the ODC mRNA increased ODC mRNA stability in these cells as well as influenced the activity of ODC. It was shown that in normal

keratinocytes HuR was unable to bind to the ODC mRNA resulting in a shorter mRNA half-life and lower ODC enzyme activity in these cells. The primary mechanism was determined to be HuR localization as HuR was primarily nuclear in normal keratinocytes and both nuclear and cytoplasmic in spindle carcinoma cells (Nowotarski and Shantz, 2010). These data suggest that HuR plays a role in regulating ODC mRNA. Based on these studies, we decided to determine whether AZ and AZI were also post-transcriptionally regulated by HuR.

Hypothesis and specific aim

Hypothesis: We will test the hypothesis that HuR binds to the 3'UTR of AZ and/or AZI in transformed cells. The binding of HuR to the 3'UTR will thus increase the mRNA stability of these transcripts.

Specific aim 1: To evaluate what contribution the stabilizing RNA binding protein HuR has on AZ mRNA stability and AZI mRNA stability.

Chapter 2

Materials and Methods

2.1 Tissue culture

C5N and A5N cell lines (a gift from Dr. Allan Balmain, University of California-San Francisco, San Francisco, CA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1% streptomycin, and 1% glutamine (Invitrogen). Flasks were incubated at 37°C in humidified air of 95% air/5% carbon dioxide.

2.2 RNA extraction

RNA was extracted using the protocol provided by the TRIzol reagent (Invitrogen). In brief, cells were treated with 200 µl chloroform and centrifuged at 12,000 rpm for 15 minutes at 4°C. The clear aqueous layer was placed into a new tube, and incubated with 3 µl of GlycoBlue (Invitrogen) and 500 µl isopropanol. This aqueous layer was incubated at room temperature for 10 minutes, and then centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was washed with 300 µl cold 75% ethanol. The sample was centrifuged again at 5,000 rpm for 5 minutes at 4°C. The ethanol was removed, and the sample was air dried for 10 minutes. The pellet was resuspended in RNAse-free water and incubated at 60°C for 10 minutes.

2.3 Analysis of RNA integrity using agarose gel electrophoresis

RNA samples were analyzed on a 1% agarose gel containing ethidium bromide. Gels were visualized by the BIO-RAD imager (BIO-RAD, Hercules, CA).

2.4 cDNA synthesis

RNA isolated using the aforementioned procedure was amplified via reverse transcription. cDNA was made by using the qScript Flex cDNA Synthesis Kit following the manufacturer's instructions (Quanta Biosciences, Gaithersburg, MD).

2.5 Biotin-labeled RNA-Binding Protein assay

Two µg of cDNA from section 2.4 was used as a template for the PCR amplification of AZ, AZI, GAPDH, and c-Myc products with a T7 promoter (Figure 2.1). GAPDH and c-Myc products were made as controls. The sense primers for each reaction contained the T7 sequence on the 5' end. These primers are listed below. The T7 sequence is:

5' GCT TCT AAT ACG ACT CAC TAT AGG GAG A 3'.

AZ (Tm= 60.2 ºC):

Forward: 5' (T7 promotor) GT GCC AGC CCT GCC CAG TGT C 3'

Reverse: 5' GCC AAT GAA CGA GAT CAC TTT ATT G 3'

AZI (Tm= 50 ºC):

Forward: 5' (T7 promotor) AC AGG CAT TAA CGC TTC TTT A 3'

Reverse: 5' TGA TAT GTA AAA TTT TAC C 3'

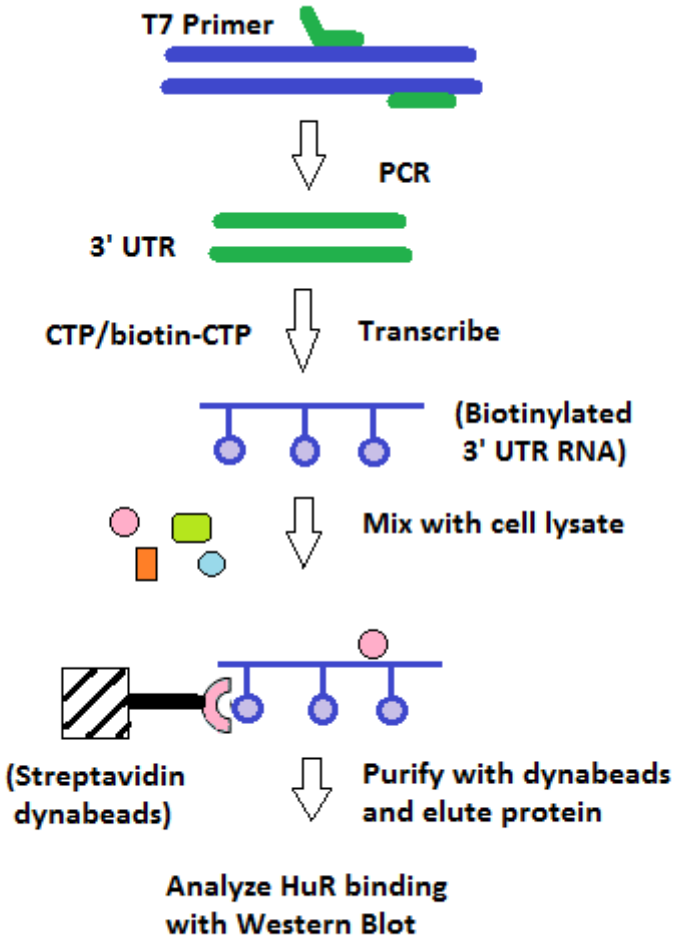

Figure 2.1 Synthetic biotin-labeled RNA-Binding Protein assay

PCR products of the 3'UTR sequences for AZ, AZI, GAPDH, and c-Myc were made containing the T7 promotor on the 5' end. The T7 polymerase was used in order to transcribe the biotin-labeled synthetic transcripts. The transcripts were incubated with cytoplasmic lysate in order for the proteins to bind with the 3'UTR sequence of the transcripts. Streptavidin dynabeads (Invitrogen) were used to the bind the biotin-labeled transcripts to pull down the 3'UTR sequences along with any bound proteins. The proteins were eluted and the binding of HuR to the transcripts was analyzed by Western blotting.

Modified from a figure by Dr. Faoud Ishmael (Penn State College of Medicine, Biochemistry dept.)

GAPDH (Tm= 62ºC):

Forward: 5’ (T7 promotor) GA AAC CCT GGA CCA CCC ACC CCA G 3’

Reverse: 5’ GGG TGC AGC GAA CTT TAT TGA TG-3’

c-Myc (Tm= 50.2ºC):

Forward: 5’ (T7 promotor) AC TGA CCT AAC TCG AGG AGG AG-3’

Reverse: 5’ GTA TTT TTT CCA ATT ATT TTA T3’

The AZ, GAPDH, and c-Myc PCR reactions were done using the MyFi 2x transcription kit as per the manufacturer’s instructions (Bioline, Taunton, MA). However, creating the AZI PCR product was problematic using this kit; therefore, the Kapa Hot Start Long Range PCR kit (Kapa Biosystems, Wilmington MA) was used to amplify this product using the following procedure and human genomic DNA:

Hold 94 C 3 min

94 C 20 sec

50 C 3 min

Repeat steps 2 and 3 for 35 cycles

50 C 10 min

The PCR products were gel purified and then used as template for the labeling procedure. The biotin-labeled 14-CTP nuclear mix contained 10 mM ATP, 10 mM GTP, 10 mM UTP, 9mM CTP, and 1 mM biotin-14-CTP. The *in vitro* transcription reaction mix used to make the biotinylated probes was as follows:

11 µl 1 µg PCR template, up to 11 µl with RNAse free water

4 µl 5x transcription buffer

2 µl 100 mM DTT

0.5 µl RNasin

1 µl Biotin-14-CTP nuclear mix

1.5 µl T7 RNA polymerase

---

20 µl Total volume

The *in vitro* mix was incubated for 2 hours at 37°C, and then treated with Dnase. The reaction mix was incubated for 15 minutes at 37°C, and then deactivated by heating the reaction to 65°C. Lastly, 25 µl TE buffer (pH 8.0) was then added to each reaction sample. The samples were passed through a G-50 column as per the manufacturer's instructions (GE, United Kingdom). RNA was precipitated and resuspended in 20 µl RNase-free 2x TENT buffer (20 mM Tris-HCL pH 8.0, 2 mM EDTA pH 8.0, 500 mM NaCl, 1% v/v Triton X-100).

The biotin-labeled synthetic RNA transcripts were incubated with 120 µg of A5 cytoplasmic lysate for 30 minutes at room temperature using an end-over-end rotator. Ten µl of streptavidin-conjugated Dynabeads (Invitrogen) were pre-washed twice with 200ul Solution A (0.1 M NaOH and 0.05 M NaCl) and once with 200 µl of solution B (0.1 M NaCl). The beads were re-suspended in 10 µl 1x TENT buffer. The RNA-protein complexes were collected via incubation with the paramagnetic Dynabeads. In brief, the biotin-labeled RNA transcripts and lysate were added to Dynabeads and incubated for 30 minutes at room temperature on an end-over-end rotator. The RNA-protein complexes were then washed once with cold PBS and then re-suspended in 45 µl 1x SDS sample buffer. The samples were heated for 10 minutes at 80°C. The RNA-protein complexes were analyzed on a 10% SDS-PAGE gel. Western blot analysis using the HuR antibody was used to assess the binding of the HuR protein to the RNA transcripts.

2.6 Whole cell lysis

Cells were extracted using RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrohosphate, 1 mM beta glycerophosphate, 1 mM Na3VO4,1 μg/ml leupeptin, 1 mM PMSF, 1X protease inhibitor cocktail) (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot analysis was performed using GAPDH and AZI antibodies.

2.7 Cellular fractionation

Cellular fractionation was done by a hypotonic solution (10 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 0.5% NP-40, and 1X protease inhibitor). Cells were pelleted at 1000 rpm for 5 min, and the pellets were resuspended in 1 volume of cold hypotonic solution. The samples were placed on ice for 5 min, dounced (10 strokes), and then pelleted again at 1000 rpm for 5 min. The supernatant was isolated as the cytoplasmic lysate. Verification of the cellular fractionation experiments occurred via Western blot analysis using Histone H3 and α/β Tubulin antibodies to determine the purity of each fraction.

2.8 Western blot analysis

All Western blots used a 10% SDS-PAGE gel. Protein was transferred onto a PVDF membrane. HuR (Santa Cruz Biotechnology), GAPDH (BioDesign International, Saco, ME), Histone H3 (Cell Signaling Technology, Danvers, MA), and α/β Tubulin (Cell Signaling Technology)

antibodies were used as described above at a 1:1000 dilution. Images were analyzed using the LI-COR FC system (LI-COR, Lincoln, NE).

Chapter 3

Results

3.1 Cell lines used in pilot studies

Two cell lines were used, one wild type cell line and one tumorigenic cell line denoted as C5N and A5 respectively (Figure 3.1). The A5 cells were derived from tumors on mice that had been treated with the multi-stage chemical carcinogenesis protocol (Zoumpourlis et al., 2003). The A5 cells were used to analyze the post-transcriptional regulation of AZ and AZI by HuR since previous work has shown that HuR is cytoplasmic in these cells. (Nowotarski and Shantz, 2010).

3.2 Verifying RNA from C5N and A5 cells

RNA was successfully isolated from the normal mouse keratinocytes and spindle carcinoma cells. The agarose gel shows the 28S and 18S rRNA bands intact suggesting that the RNA used to make cDNA was not degraded (Figure 3.2).

3.3 Verifying PCR products for AZ, AZI, GAPDH, and c-Myc

In order to create the T7 biotin probes for the pulldown assays, the 3'UTR of AZ, AZI, GAPDH, and c-Myc needed to be amplified. A PCR product which correlated with the full length AZ 3'UTR, approximately 280 bp, was obtained. After several failed attempts a PCR product that correlated to AZI 3'UTR, approximately 2800 bp, was obtained as well. PCR

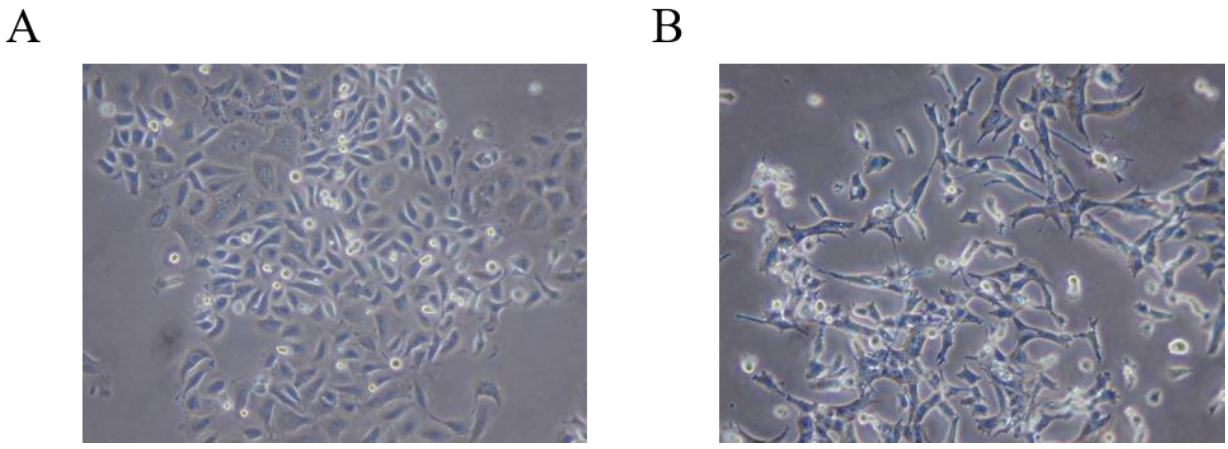

Figure 3.1 Normal mouse keratinocytes (C5N) and spindle carcinoma cells (A5)

(A) C5N cells. C5N cells are normal mouse keratinocytes. (B) A5 cells are spindle carcinoma cells. A5 cells were used in our pilot studies to analyze AZ and AZI post-transcriptional regulation in non-melanoma skin cancer development.

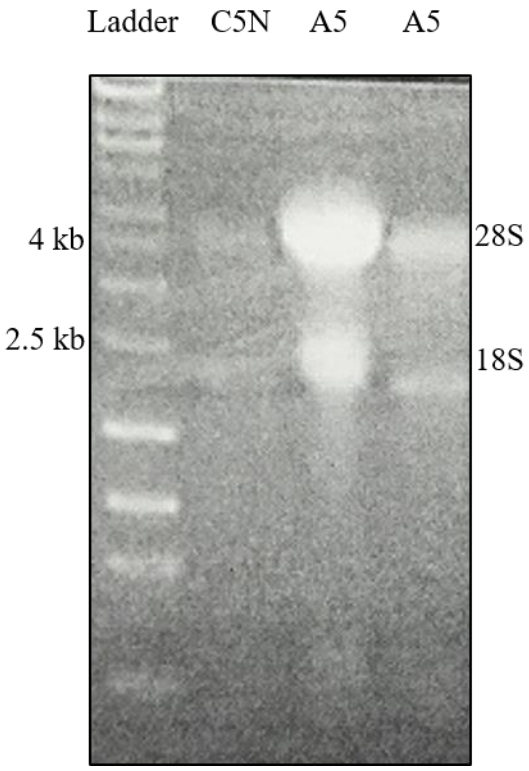

Figure 3.2 A5 and C5N RNA integrity

A 1% agarose gel was used to indicate RNA integrity. BIOLINE Hyperladder 1kb Plus (Bioline) was used to indicate size. C5N and A5 RNA both produced bands at 3.8kb and 1.8kb. The bands at 3.8kb correlates to 28S rRNA. The bands at 1.8kb correlate to 18S rRNA. There are also faint bands at 0.25kb which correlate to tRNA.

products that correlated to the GAPDH 3'UTR and c-Myc 3'UTR, 400 bp and 150 bp respectively, were also obtained. These PCR products were then used as a template for the biotin labeling of the 3' UTRs of AZ, AZI, GAPDH, and c-Myc (Figure 3.3).

3.4 Whole cell lysate analysis of C5N and A5 cells

In order to conduct the biotin-labeled RNA-protein binding assay, we needed to ensure that AZ and AZI were present in the cell lines. A5 and C5N cells were lysed using 1X RIPA buffer in order to show that AZI is indeed present within both cell types (Figure 3.4). GAPDH was used as a loading control to show that the same amount of protein was loaded from both cell lines. There was no change in the amount of AZI protein from the C5N cells to the A5 cells (data not shown). Moreover, previous results demonstrated both C5N and A5 cells contained similar levels of AZ protein (data not shown).

3.5 Cellular fractionation of A5 cells

A cytoplasmic fraction was isolated from A5 in order to verify that HuR was indeed located within the cytoplasm (Nowotarski and Shantz, 2010). Western blot analysis also verified that the cytoplasm isolation was not completely pure since there were some nuclear proteins present in the cytoplasmic fraction. In any case, an enrichment of HuR was shown in our used fraction, indicating this fraction was good to use to test HuR's ability to bind to the biotinylated probes (Figure 3.5).

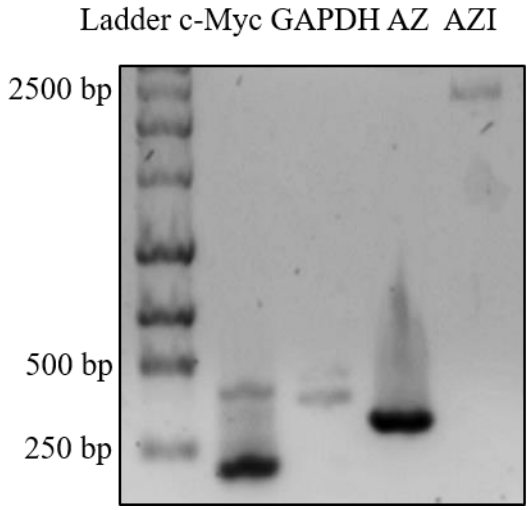

Figure 3.3 PCR products for AZ, AZI, GAPDH, and c-Myc

A BIOLINE Hyperladder 1kb Plus (Bioline) was used to indicate PCR product size on a 1% agarose gel. A band is shown at 240 bp suggesting that the 3'UTR of c-Myc was obtained. The band at 400 bp suggests that the 3' UTR of GAPDH was obtained. The band at 280 bp suggests that the 3'UTR of AZ was obtained. The band at 2800 bp suggests that the 3'UTR of AZI was obtained.

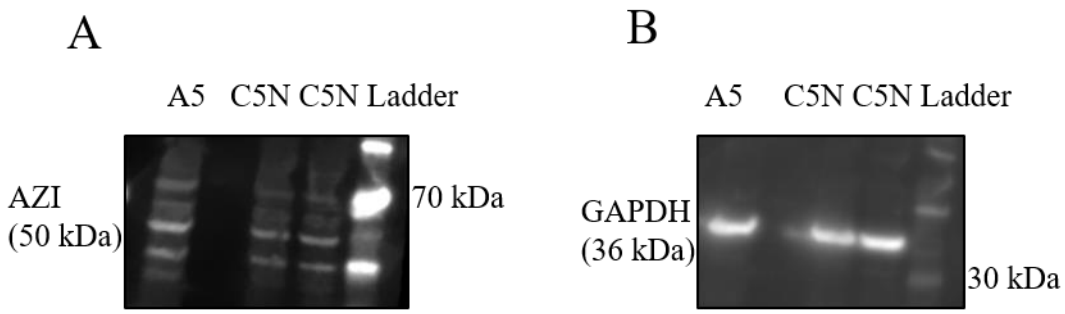

Figure 3.4 Whole cell lysate Western blot

(A) Whole cell lysate Western blot with the AZI antibody. Bands at 50 kDa for the C5N and A5 whole cell lyses verify that AZI is located within the cells. (B) Whole cell lysate Western blot with the GAPDH antibody. Bands at 36 kDa for the C5N and A5 whole cell lyses were used as a control. The amount of AZI protein was quantitated using the LI-COR imager (LI-COR).

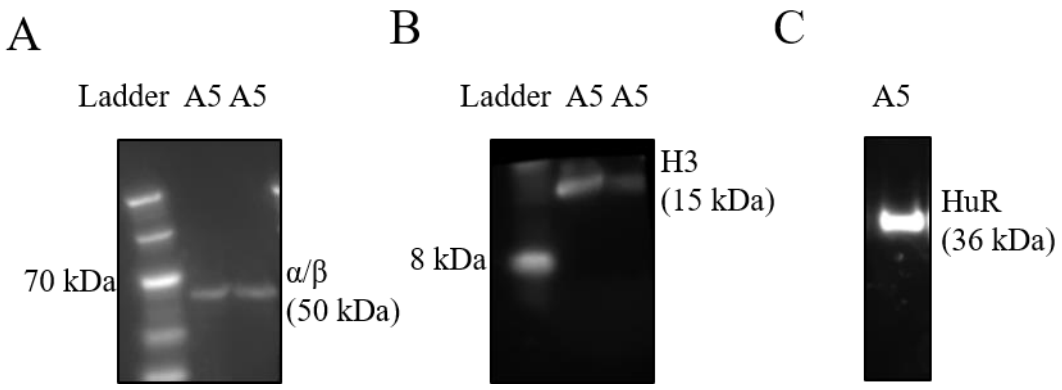

Figure 3.5 Cellular fractionation Western blot

(A) Western blot for 20 µg A5 cytoplasmic lysate using α/β Tubulin (50 kDa) antibody. (B) Western blot for 20 µg A5 cytoplasmic lysate using the Histone H3 (15 kDa) antibody. (C) Western blot for 20 µg A5 cytoplasmic lysate using the HuR (36 kDa) antibody.

3.6 Biotin pulldown of AZ, AZI, GAPDH, and c-Myc 3'UTR in A5 cells

A pilot study was conducted to test whether HuR was able to bind to the 3'UTR of AZ and AZI in the A5 cell line. GAPDH was used as a negative control since there are no data to suggest that HuR binds to the 3'UTR of GAPDH. Previous data has shown that the 3'UTR of c-Myc does bind to HuR, thus c-Myc was used as a positive control. Unfortunately, our initial pulldown did not yield results. The positive control, c-Myc, did not even show binding to HuR, suggesting that our probes were of poor quality or even degraded (data not shown).

Chapter 4

Discussion

In this work we show the initial results needed to obtain PCR products for AZ, AZI, c-Myc and GAPDH. These PCR products were subsequently used to create biotin-labeled probes for each mRNAs 3'UTR to be used in a pulldown experiment to show whether or not HuR is able to bind to these mRNAs.

Spindle cell carcinoma cells (A5) were used in these experiments because it has previously been shown that HuR is localized to the cytoplasm in these cells (Nowotarski and Shantz, 2010). Thus, SCCs were the model of choice to compare AZ and AZI post-transcriptional regulation by HuR (Figure 3.1).

PCR products were produced for the 3'UTR of AZ, GAPDH, c-Myc, and AZI (Figure 3.3). A band was formed at 280 bp, which suggests that the 3'UTR of AZ was properly amplified. A band was formed at 2800 bp suggesting that the 3'UTR of AZI was properly amplified. Bands at 240 bp and 400 bp suggest that the 3'UTRs of c-Myc and GAPDH were properly amplified respectively. Cytoplasmic fractionation of A5 cells revealed that HuR is located within the cytoplasm of these cells in accordance with previous findings (Figure 3.4). The PCR products were utilized for the biotin-labeled RNA-protein binding assay in order to see if HuR would bind to the 3'UTR of AZ and AZI. Whole cell lysis of A5 and C5N cells revealed that both AZ and AZI were located within the cells with the same amount of protein in each cell type.

The biotin-labeled RBP assay was uninterpretable. This could be due to the probes, which may have been degraded, because we see that HuR was indeed present in the cytoplasmic

lysate used (Figure 3.4). To continue this project, more work could be done in order to properly make the probes and ensure their stability and integrity.

The work in this thesis has produced all of the necessary elements needed to analyze the interaction of HuR with the 3'UTRs of AZ and AZI. The pulldown assays unfortunately were unsuccessful, but more work can be done to better understand the interaction of HuR with AZ and AZI 3'UTRs. An endogenous pulldown could be performed to see if HuR does indeed bind to the AZ 3'UTR and/or AZI 3'UTR in tumorigenic cells to increase mRNA stability. Moreover, other RNA binding proteins such as Vg1RBP could be studied (Kalous et al., 2014). Also, miRNAs could be used to study their effects on AZ and AZI stability, such as miR-433 suppressing AZI expression (Li et al., 2013)

Thus, the continuation of these initial studies will provide a proof-of-concept focusing on AZ and AZI in NMSC. Polyamine pathway proteins, such as ODC, have already been well documented with NMSC. AZ and AZI could provide an additional target of NMSC regulation, and in turn a potential cancer therapy.

AZ and AZI are endogenous regulators of ODC within the polyamine pathway. If AZ or AZI are overexpressed or inhibited, major changes to ODC can occur. Such alterations to ODC expression could alter the entire polyamine pathway. Changes, such as inhibition and overexpression of proteins, such as ODC, in the polyamine pathway have been linked to cancer, specifically NMSC progression. As NMSC is the most commonly diagnosed cancer in the United States, it is increasingly important to research and discover possible targets for prevention and/or therapy. Studying AZ and AZI mechanisms in the polyamine pathway is invaluable to better understand and treat NMSC.

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