The Pennsylvania State University
The Graduate School
Intercollege Graduate Degree Program in Genetics

STUDIES ON THE POST-TRANSCRIPTIONAL REGULATION OF ORNITHINE
DECARBOXYASE IN NON-MELANOMA SKIN CANCER

A Dissertation in
Genetics
by
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ABSTRACT

Ornithine decarboxylase (ODC) is the first enzyme in the polyamine biosynthetic pathway. Under normal physiological conditions, polyamine content and ODC enzyme activity are highly regulated. Induction of ODC activity is an early step in neoplastic transformation. The studies described here use normal mouse keratinocytes (C5N cells), and spindle carcinoma cells (A5 cells) to explore the regulation of ODC synthesis in non-melanoma skin cancer (NMSC) development. We see a marked increase in ODC enzyme activity in A5 cells compared to C5N keratinocytes, which correlates with ODC mRNA stabilization. These data suggest that ODC is post-transcriptionally regulated. We sought to investigate whether the ODC transcript interacts with the RNA binding protein HuR, which is known to bind to and stabilize its target mRNAs. We show that HuR is able to bind to the ODC 3'UTR in A5 cells but not in C5N cells. Immunofluorescence results reveal that HuR is present in both the nucleus and cytoplasm in A5 cells, while it is strictly nuclear in C5N cells. Knockdown experiments in A5 cells showed that when HuR is depleted ODC RNA becomes less stable and ODC enzyme activity decreases. Together, these data support the hypothesis that HuR plays a causative role in ODC upregulation during NMSC development by binding to and stabilizing the ODC transcript. Additionally, we show that the destabilizing RNA binding protein tristetraprolin (TTP) binds to the ODC 3'UTR. Moreover, using mouse embryonic fibroblasts (MEFs) in which TTP is deleted, we find that the ODC transcript is stabilized. Finally, we show that the 3'UTR and 5'UTR of ODC contain cis-acting negative regulatory elements. We demonstrate that luciferase activity is significantly lower in both C5N and A5 cells transfected with vectors that contain either the ODC 3’UTR or 5’UTR when compared to cells transfected with vectors that contain neither UTR. Furthermore, the classical adenosine- and uracil- rich element (ARE) within the ODC 3’UTR acts as a
negative regulatory element. The data in this thesis show for the first time that the ODC transcript is regulated by two trans-acting factors, HuR and TTP.
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<td>3'UTR</td>
<td>3' untranslated region</td>
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<tr>
<td>4E-BP1</td>
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<td>5'cap</td>
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<td>5'UTR</td>
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<td>AICAR</td>
<td>5-Aminoimidazole-4-carboxamide ribonucleoside</td>
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<td>DAG</td>
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<tr>
<td>HNS</td>
<td>HuR nucleocytoplasmic shuttling</td>
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</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of Rapamycin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NMSC</td>
<td>non-melanoma skin cancer</td>
</tr>
<tr>
<td>ODC</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PABP</td>
<td>Poly(A) tail binding protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAN</td>
<td>Proteosome-activating nucleotidase</td>
</tr>
<tr>
<td>PAO</td>
<td>Polyamine oxidase</td>
</tr>
<tr>
<td>PARN</td>
<td>Poly(A)-specific ribonuclease</td>
</tr>
<tr>
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<td>Processing body</td>
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</tr>
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<td>Protein kinase C</td>
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<td>Protein phosphatase 2</td>
</tr>
<tr>
<td>PUVA</td>
<td>psoralen + UVA</td>
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<tr>
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</tr>
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<tr>
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<td>Regulatory associated protein of mTOR</td>
</tr>
<tr>
<td>RBP</td>
<td>RNA binding protein</td>
</tr>
<tr>
<td>REMSA</td>
<td>RNA electromobility shift assay</td>
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</table>
Rheb  Ras homolog enriched in brain
RICTOR  Rapamycin insensitive companion of mTOR
RIE  rat intestinal epithelial
RNAi  RNA interference
RNP  ribonucleoprotein
RRM  RNA recognition motif
SCC  squamous cell carcinoma
Sencar  sensitivity to carcinogenesis
SIN1  SAPK interacting protein 1
SMO  Spermine oxidase
SNP  single nucleotide polymorphism
SSAT  Spermidine/spermine-N$_1$-acetyltransferase
TIS11  TPA inducible sequence 11
TNF-α  Tumor necrosis factor α
TPA  12-O-Tetradecylphorbol-13-acetate
TRAMP  transgenic adenocarcinoma of the mouse prostate
TRN  Transportin protein
TSC  Tuberous sclerosis complex
TSP1  Thrombospondin 1
TTP  Tristetraprolin
UTR  untranslated region
UVR  ultraviolet radiation
VEGF  Vascular endothelial growth factor
XRN1  Exoribonuclease
CHAPTER 1

Introduction

1.1 Non-melanoma skin cancer (NMSC)

1.1.1 Overview

Non-melanoma skin cancer (NMSC) is the most common malignancy in the United States, accounting for approximately 40% of all diagnosed cancers [Reviewed in (Bowden 2004)]. In fact, according to recent studies it is projected that over 2 million Americans are treated for NMSC each year (Rogers, Weinstock et al. 2010).

There are two major forms of NMSC, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) which account for 80% and 16% of all diagnosed skin cancers respectively [Reviewed in (Bowden 2004)]. BCCs are slow-growing and rarely metastasize, whereas SCCs are more invasive and metastasize at a higher frequency [Reviewed in (Bowden 2004; Madan, Lear et al. 2010)]. Although BCCs and SCCs are rarely lethal, with SCCs accounting for approximately 2,500 cancer deaths annually, these forms of NMSC are associated with considerable morbidity and healthcare costs (Dermatology 2008). It has been estimated that among those on Medicare, NMSC is the fifth most costly cancer to treat overall; and in 2004 the cost to treat NMSC was over 1 billion dollars (Bickers, Lim et al. 2006; Rogers, Weinstock et al. 2010). As with most cancers, NMSC prevalence increases with age. It has been estimated that 80% of NMSC cases occur in individuals aged 60 or older [Reviewed in (Madan, Lear et al. 2010)]. However, another estimate has shown NMSC to be on the rise in young adults (Christenson, Borrowman et al. 2005). Overall, a 3-8% increase in NMSC incidence has been reported worldwide since 1960 [Reviewed in (Madan, Lear et al. 2010)]. Experts speculate that the rise in NMSC incidence is due to increased lengths of time spent
outdoors, the use of tanning beds, and the depletion of the protective ozone layer, which filters ultraviolet radiation (Karagas, Stannard et al. 2002; Bowden 2004).

1.1.2 Ultraviolet radiation (UVR) and NMSC

Like melanoma, the primary risk factor for NMSC is exposure to UV radiation (UVR) (Armstrong and Kricker 1993; Bowden 2004). UV light from the sun is categorized into three forms according to wavelength ($\lambda$): UVA, UVB, and UVC. UVA ($\lambda=320-400$ nm) and UVB ($\lambda=280-320$ nm) reach the Earth’s surface, whereas UVC ($\lambda=200-280$ nm) is filtered by the protective ozone layer [Reviewed in (Bowden 2004)]. Studies pertaining to skin carcinogenesis in mice have shown that UVA and UVB are complete carcinogens acting as initiating and promoting agents. UVA, however, is a weak complete carcinogen, functioning as a more potent tumor promoting agent than initiating agent (Willis, Menter et al. 1981; Strickland 1986; Bowden 2004; Runger 2007).

In terms of the pathophysiology of NMSC, UVR has been shown to cause direct DNA damage by initiating the formation of cyclobutane dimers, 6-4 photoproducts, cytosine photohydrates, DNA cross-links, and DNA double-strand breaks [Reviewed in (Bowden 2004)]. If such DNA adducts are not repaired by nucleotide excision repair, then classical $\text{CC} \rightarrow \text{TT}$ and $\text{C} \rightarrow \text{T}$ mutations can ensue during replication. These UVR mutation signatures have been detected in the $p53$ gene, a gene that is mutated at a high frequency in both BCC and SCC tumors (Ziegler, Leffell et al. 1993). Additionally, the Harvey-ras (Ha-ras) gene is mutated and activated in NMSC (van der Schroeff, Evers et al. 1990; Pierceall, Goldberg et al. 1991). A study conducted on psoriasis patients who were treated with psoralen, a natural product found in fig and celery which absorbs UV, and UVA (PUVA) revealed that the Ha-ras gene was mutated in 76% of SCC tumor samples (Kreimer-Erlacher, Seidl et al. 2001). Furthermore, other genes such as Cox-2 have been shown to be induced by UVR (An, Athar et al. 2002; Zhang and Bowden 2008).
To date, the best treatment for NMSC is surgery, which often leaves scarring. Few effective chemoprevention methods are available for NMSC. The best methods of prevention include: the application of sun block, limiting time outdoors, and wearing protective clothing. Thus, NMSC is an attractive cancer model to study, as understanding underlying principles of this disease will lead to the advancement of better prevention and treatment strategies.

1.2 Multi-stage chemical carcinogenesis model for skin cancer

1.2.1 Overview

The multi-stage chemical carcinogenesis model is the most extensively used model for studying chemically induced neoplasia. Furthermore, this model, originally used to study cancer in rodents, has been confirmed as a valid tool for studying human epithelial cancers, as humans are naturally exposed to low doses of carcinogens and tumor promoting agents [Reviewed in (DiGiovanni 1992)]. Thus, the use of the multi-stage chemical carcinogenesis model in the studies described herein is not only valid for studying the development of NMSC in mice, but is also applicable for studying the process of skin carcinogenesis in humans. Furthermore, it has been shown that the multi-stage chemical carcinogenesis model for skin cancer is physiologically relevant because it induces similar mutations to those produced by UVR exposure. For example, during multi-stage chemical carcinogenesis Ha-ras is mutated at a high frequency at codon 61 (Quintanilla, Brown et al. 1986; DiGiovanni 1992). Similarly, during UVR exposure the Ha-ras gene is mutated, but often at codon 12 (Pierceall, Goldberg et al. 1991; Kreimer-Erlacher, Seidl et al. 2001). Each of these mutations, however, results in the constitutive activation of Ras-dependent downstream signaling pathways.

The mouse multi-stage chemical carcinogenesis model for skin cancer is well established and is one of the best in vivo models for studying tumorigenesis because it
allows one to follow the development of skin tumors from the initiation stage through the promotion and progression stages. This model also provides the opportunity to study the effects of chemoprevention agents, agents used to delay or prevent the development of cancer, at various stages during skin carcinogenesis.

1.2.2 History

Early predecessors of the multi-stage chemical carcinogenesis model date back to the 1910s when Yamagiwa and Ichikawa painted rabbit skin with coal tar to induce tumors [Reference therein (Marks and Furstenberger 1986)]. In the 1920s Deelman discovered that wounding after the application of a carcinogenic tar caused the development of skin tumors in mice [Reference therein (DiGiovanni 1992)]. Friedewald and Rous were the first to define the terms “initiation” and “promotion” when describing skin carcinogenesis. Using rabbits, they demonstrated that tumor cells were initiated by a single treatment of the carcinogen 3-methylcholanthrene (MCA). The initiated cells could be promoted into tumors after subsequent treatment of the skin with agents that were not able to cause neoplastic transformation when applied by themselves (i.e. turpentine and chloroform) (Friedewald and Rous 1944). In the 1940s the initiation and promotion approach, a procedure still used today to study skin carcinogenesis, was established. This occurred after the discovery that croton oil was a potent promoting agent. Tumor initiation was achieved by treating mice with a sublethal dose of a carcinogen, most commonly 7, 12-dimethylbenz[a]anthracene (DMBA). Promotion occurred via the chronic application of croton oil (Mottram 1944; Berenblum and Shubik 1947). The use of this model revealed that initiation is irreversible and that the sequence of initiation and promotion was not interchangeable (Berenblum and Shubik 1949; Berenblum and Haran 1955). The discovery that phorbol diesters, particularly 12-O-Tetradecynlyphorbol-13-acetate (TPA), are the active ingredients of croton oil ultimately led to the development of the multi-stage chemical carcinogenesis model used
today in which an initiation agent is first applied to the mouse skin followed by repeated applications of TPA (Boutwell 1964; Van Duuren and Orris 1965; Hecker 1971).

1.2.3 Initiation

Figure 1.1 provides a schematic depiction of the multi-stage chemical carcinogenesis model. Initiation occurs with a single application of a carcinogen. Often, the polycyclic aromatic hydrocarbon, DMBA, is applied topically as the initiating agent. The initiating agent causes mutations to regulatory genes (i.e. Ha-ras and p53) in epidermal keratinocytes by interacting with DNA and forming N⁶-dAdo DNA adducts [Reviewed in (DiGiovanni 1992)]. One hallmark of the multi-stage chemical carcinogenesis model is that an A→T transversion frequently occurs in the Ha-ras oncogene at codon 61 after DMBA application (Quintanilla, Brown et al. 1986). Ha-ras mutations can be observed in the epidermis 1 week after DMBA application (Nelson, Futscher et al. 1992). The importance of the Ha-ras mutation and subsequent activation of the Ras pathway in the development of skin tumors is highlighted in two studies. Balmain et al. demonstrated that Ha-ras expression was increased in mouse papillomas when compared to normal epidermis (Balmain, Ramsden et al. 1984). Furthermore, Spalding et al. showed mice that were not treated with DMBA, or any other primary carcinogen, but that overexpressed v-Ha-ras developed skin tumors after treatment with promoting agents, suggesting that Ras activating mutations are early and critical events for the development of skin tumors (Spalding, Momma et al. 1993). It is hypothesized that the DMBA induced mutations occur within the stem cells of keratinocytes [Reviewed in (Morris 2004)]. Currently, there are two proposed regions in which these stem cells reside—the interfollicular epidermis and the bulge region of the hair follicle (Abel, Angel et al. 2009).
Figure 1.1. The multi-stage chemical carcinogenesis model for NMSC.

Initiation occurs with the application of a subcarcinogenic dose of the initiating agent, often DMBA. Initiation causes the formation of DNA adducts, and leads to mutations in critical genes in keratinocyte stem cells. During promotion, repeated applications of a tumor promoting agent, often TPA, leads to the formation of papillomas. Few papillomas convert to squamous cell carcinomas which occur after 20 weeks of TPA application. Conversion of papillomas to carcinomas requires additional genetic events: aneuploidy, loss of heterozygosity, and dysplasia. Rarely, carcinomas will progress to become invasive and metastatic. H&E sections are shown, and represent important stages in the multi-stage chemical carcinogenesis model. Normal skin, hyperplastic epidermis, papillomas, and squamous cell carcinomas are shown.

Source: (Abel, Angel et al. 2009).
1.2.4 Promotion

During tumor promotion, initiated cells that have acquired a growth advantage clonally expand to form papillomas. Promotion occurs after repeated applications of tumor promoting agents or wounding of the skin. Such applications with tumor promoting agents often lead to epidermal hyperplasia with increased epidermal thickness (Kruszewski, Naito et al. 1989). A well studied tumor promoting agent, the phorbol ester TPA, is classically used in the multi-stage chemical carcinogenesis model. The hydrophobicity of the acyl chain in phorbol esters is critical for their tumor promoting ability and phorbol esters have been shown to increase mRNA and protein synthesis [Reviewed in (Griner and Kazanietz 2007); Reference therein (DiGiovanni 1992)].

TPA also has the ability to bind to and activate Protein Kinase C (PKC) [Reviewed in (Griner and Kazanietz 2007)]. This occurs because TPA is an analogue of, and thus mimics the actions of, PKC’s natural activator diacylglycerol (DAG) (Figure 1.2). PKC is a family of serine/threonine kinases that regulate cell proliferation, apoptosis, survival, and migration. Ten isoforms of the protein have been identified with diverse functions. PKC α, βI, βII, and γ are denoted as classical isoymes and are all activated by TPA in a calcium dependent manner. The novel PKC isoymes, δ, ε, θ, and η are responsive to TPA in a calcium independent manner, and the atypical PKC isoymes ζ and λ have been shown to be non-responsive to TPA [Reviewed in (DiGiovanni 1992; Griner and Kazanietz 2007)]. In brief, PKC activation occurs in two steps. PKC undergoes a process of maturation prior to its activation involving phosphorylation of its activation loop by Phospholipid-dependent Kinase 1 (PDK1). This is followed by autophosphorylation which leads to the mature PKC enzyme. The mature, phosphorylated PKC is then released into the cytosol where it is kept in an inactive state. Upon stimulation of receptors that increase intracellular DAG and calcium (i.e. plasma membrane receptors), PKC is tethered to the membrane via calcium binding
**Figure 1.2.** Protein Kinase C (PKC) maturation and activation.

The process of maturation occurs prior to activation of PKC. Phospholipid-dependent Kinase 1 (PDK1) phosphorylates the activation loop of PKC which is subsequently followed by autophosphorylation of PKC resulting in a mature PKC. The mature PKC is kept in an inactive state. After stimulation of receptors and increases in both intracellular diacylglycerol (DAG) and calcium, PKC is tethered to the membrane through calcium binding to the C2 domain and DAG binding to the C1 domain of PKC. A conformational change in PKC occurs allowing PKC to activate downstream targets.

Source: (Griner and Kazanietz 2007).
to the C2 domain of the enzyme. DAG binding to the C1 domain results in the interaction of PKC with the cell membrane, leading to a conformational change. This allows substrate binding, phosphorylation, and activation of PKC’s downstream targets [Reviewed in (Newton 2003; Griner and Kazanietz 2007)].

PKCε overexpressing mice have been shown to develop SCCs after DMBA-TPA treatment or after repeated UV exposure. Additionally, in cell culture, PKCε has been defined as an oncogene due its transforming characteristics. Overexpressing PKCε in NIH-3T3 mouse fibroblasts led to increased growth rates, anchorage independent growth, and tumor development in nude mice [Reviewed in (Verma, Wheeler et al. 2006)]. Moreover, PKCε overexpression in the skin sensitizes mice to develop only SCCs [Reviewed in (Verma, Wheeler et al. 2006)]. In contrast, PKCδ overexpression in the skin suppressed the formation of both papillomas and SCCs in mice that were treated with DMBA and TPA (Reddig, Drekschmidt et al. 1999). These data show that the overexpression of some PKC isoforms can be linked to NMSC. The exact mechanisms whereby TPA induces skin tumors are still being investigated.

1.2.5 Progression

Progression is the conversion of benign papillomas to carcinomas. During progression, chromosomal abnormalities, such as aneuploidy, develop and additional genetic mutations occur. The carcinomas that may subsequently develop can be both invasive and metastatic; approximately 15-35% of CD-1 mice with one or more carcinomas display metastases in either the lungs or lymphatic system (Hennings, Spangler et al. 1986). The rate of spontaneous progression of papilloma to carcinoma is dependent on the genetic background of the mice used. For example, 10% of papillomas convert to SCCs in SENsitivity to CARcinogenesis (SENCAR) mice; whereas FVB/N mice exhibit SCC conversion at a 50% rate (Hennings, Glick et al. 1993). The subsequent conversion of SCCs to spindle carcinomas is a rare event.
The multi-stage chemical carcinogenesis model for skin cancer is one of the best in vivo models for studying NMSC, and has aided in understanding the underlying mechanisms of this disease. Specifically, data from studies using the multi-stage chemical carcinogenesis model have suggested that polyamines, namely putrescine, and the enzyme ornithine decarboxylase (ODC) are important in the development of NMSC. TPA has been shown to induce the expression of ODC (O'Brien, Simsiman et al. 1975; O'Brien 1976; O'Brien, Lewis et al. 1979). Furthermore, ODC enzyme activity has been found to be increased in the tumors of DMBA-TPA treated mice (O'Brien, Simsiman et al. 1975; O'Brien 1976). Thus, a positive correlation between ODC induction and tumor development has been established.

1.3 The polyamine pathway

1.3.1 Overview

Polyamines were first discovered in 1678 by Antonie van Leeuwenhoek who isolated what he deemed “crystals” from human semen [Reference therein (Wallace, Fraser et al. 2003)]. However, it was not until 1924 that the chemical structure of these crystals was deduced. The molecules were later named spermidine and spermine (Dudley, Rosenheim et al. 1924).

Polyamines, small ubiquitous polycations are essential for normal cell growth and development (Fozard, Part et al. 1980; Pegg 1988; Pendeville, Carpino et al. 2001). They are present in every organism except Methanobacteriales and Halobacteriales—two families of Archaea (Scherer and Kneifel 1983; Chen and Martynowicz 1984). Their positive charge allows polyamines to interact with polyanionic macromolecules including DNA, RNA, proteins, and acidic phospholipids (Schuber 1989; Wallace, Fraser et al. 2003). Polyamines bind strongly to the anionic backbone of DNA due to their flexibility and charge distribution (Igarashi, Sakamoto et al. 1982; Matthews 1993; Wallace, Fraser...
et al. 2003). Through the binding of polyamines to DNA, RNA, and proteins, gene expression can be influenced by the polyamine pool.

1.3.2 Polyamine metabolism and catabolism

Polyamine metabolism is a highly regulated process and consists of multiple interdependent enzymatic reactions (Figure 1.3). In mammalian cells, polyamines are synthesized from the amino acids L-methionine and L-arginine. L-arginine is metabolized into L-ornithine via arginase. L-ornithine is then decarboxylated by the enzyme ornithine decarboxylase (ODC) to produce the diamine putrescine. Putrescine is subsequently converted to the higher polyamines spermidine and spermine. Aminopropyl groups are donated by S-adenosylmethionine (AdoMet) after L-methionine is converted into AdoMet by methionine adenosyltransferase (MAT). The decarboxylation of AdoMet by S-adenosylmethionine decarboxylase (AdoMet-DC) creates decarboxylated AdoMet which donates aminopropyl groups to form spermidine and spermine through the enzymes spermidine synthase and spermine synthase respectively.

Additionally, the intracellular polyamine pools are also regulated via a catabolic pathway. In this, spermine and spermidine are acetylated by spermidine/spermine-\textit{N}^1\text{-acetyltransferase} (SSAT), which uses acetyl-CoA to produce \textit{N}^1\text{-acetyl}spermine and \textit{N}^1\text{-acetyl}spermidine. The acetylated polyamines become substrates for the FAD-dependent polyamine oxidase (PAO) which catalyzes their conversion back to both spermidine and putrescine. Alternatively, spermine oxidase (SMO) is able to oxidize non-acetylated spermine to spermidine creating the byproducts H$_2$O$_2$ and aldehyde 3-aminopropanol. Upon back conversion, putrescine and \textit{N}^1\text{-acetyl}spermidine can be exported out of the cell by the diamine exporter (DAX) and then eradicated in urine [Reviewed in (Wallace 2000; Pegg, Feith et al. 2003; Gilmour 2007)].
Figure 1.3. The polyamine metabolic and catabolic pathways.


Source: (Pegg, Feith et al. 2003).
Under normal physiological conditions, the polyamine content of the cell remains constant due to changes in the key enzymes described above. For example, when levels of spermine and spermidine are elevated the enzymes SSAT and PAO are induced in a polyamine-dependent manner. The induction of these catabolic enzymes allows the cell to maintain polyamine homeostasis. Moreover, elevated levels of the polyamines can affect ODC content through transcription, translation, and protein degradation changes [Reviewed in (Pegg 2009)]. In fact, the endogenous inhibitor of the ODC protein, antizyme 1 (AZ1), is translated in a polyamine-dependent manner (Nilsson, Koskiemi et al. 1997). This process is further described in Section 1.4.4. In addition, AdoMetDC protein half-life is increased when polyamine levels are decreased (Kahana 2007). Thus, the intracellular levels of polyamines are tightly regulated in the cell through complex metabolic, catabolic, and poorly understood transport mechanisms. This supports the importance of polyamines for normal cell growth and development. Moreover, aberrant polyamine expression is associated with numerous physiological conditions. For example, decreased levels of polyamines, specifically spermine, are associated with Snyder-Robinson syndrome, an X-linked condition associated with mental retardation, skeletal defects, and hypotonia (Becerra-Solano, Butler et al. 2009). Increased intracellular polyamine pools have been associated with enhancing beta-andrenergic induced cardiac hypertrophy and cancer (Shantz, Feith et al. 2001; Gerner and Meyskens 2004).

1.4 Ornithine decarboxylase (ODC)

1.4.1 Overview

ODC is a critical enzyme required for normal cell growth and development. This is demonstrated by the fact that knockout of ODC in mice is lethal in utero (Pendeville, Carpino et al. 2001). The first record of putrescine conversion by the decarboxylation of
ornithine occurred in 1940 by Gale who did extensive studies on amines in bacteria (Gale 1940). He reported the presence of arginine and ornithine decarboxylases in *E. coli* cultures. It was not until 1968 that mammalian ODC was discovered simultaneously in three separate laboratories (Janne and Raina 1968; Pegg and Williams-Ashman 1968; Russell and Snyder 1968). Since then, defining the role and regulation of the ODC homodimer has been the focus of many investigators. Thus far, ODC has been shown to be regulated by several mechanisms including transcription, translation, and protein degradation.

### 1.4.2 Transcriptional regulation of ODC

The rate of ODC transcription is influenced by hormones, growth factors, and tumor promoting agents (Katz and Kahana 1987; Verma, Hsieh et al. 1988). The promoter region of the *Odc* gene contains numerous sequences that are homologous to known transcription factor binding sites including: a cAMP response element, CAAT and LSF motifs, AP-1 and AP-2 sites, Sp1 sites, and a TATA box (Abrahamsen, Li et al. 1992; Li, Abrahamsen et al. 1994; Zhao and Butler 2001; Pegg 2006). One well established mechanism of *Odc* transcriptional regulation involves the oncogenic transcription factor c-Myc, which mediates proliferation, differentiation, and apoptosis.

The human *Odc* promoter region contains two E-boxes housing the sequence CACGTG (Bello-Fernandez, Packham et al. 1993). In the mouse *Odc* promoter, an additional CACGTG motif has been identified on the 5\' flanking region (Auvinen, Jarvinen et al. 2003). This consensus sequence is the binding site for the c-Myc:Max active transcription factor complex. c-Myc, a basic helix-loop-helix-leucine-zipper transcription factor (b-HLH-Zip), requires dimerization with Max, a small b-HLH-Zip protein, in order to become activated and bind to the CACGTG recognition sequence (Blackwood, Luscher et al. 1992; Auvinen, Jarvinen et al. 2003). Upon activation, the c-Myc:Max complex binds to its consensus sequence and increases the ODC message
(Bello-Fernandez, Packham et al. 1993). However, in quiescent cells, the inactive Mnt:Max antagonist complex can bind to the CACGTG sequence and inhibit Odc transcription (Figure 1.4) (Nilsson, Maclean et al. 2004). Furthermore, in $Apc^{Min/+}$ mice, a model in which a wild-type allele of $Apc$ is lost and c-Myc expression is increased, ODC mRNA expression is induced in both the colon and small intestine (Erdman, Ignatenko et al. 1999).

In addition to regulation by c-Myc, Odc transcription can be modulated by TPA and Ras activation (Gilmour, Avdalovic et al. 1985; Holtta, Sistonen et al. 1988; Zhao and Butler 2001). The tumor promoting agent TPA has been shown to induce Odc transcription. Zhao and Butler demonstrated that the rat Odc promoter is induced by TPA, and specifically showed that the nucleotide -20 to -10 region was necessary for the TPA response (Zhao and Butler 2001). This region falls between the TATA box and transcription start site of the Odc gene, and is identical between the rat and mouse. Furthermore, the region contains the same thymine content as the human Odc gene, suggesting conserved functionality. However, a canonical TPA response element has not been identified in the Odc promoter region, and the mechanism of ODC transcription induction in response to TPA remains elusive. Activation of Ras, a known regulator of growth and differentiation, was shown to modestly enhance Odc transcription in NIH-3T3 cells that overexpressed Ha-ras (Holtta, Sistonen et al. 1988; Shantz 2004). This transcriptional response was shown to be mediated through the Raf/MEK/ERK pathway. These examples demonstrate how ODC is regulated at the level of transcription and how the enzyme’s transcription is induced by oncogenic factors.

1.4.3 Translational regulation of ODC

The translation efficiency of ODC is influenced not only by the structure of the ODC mRNA, but also by the availability of translation factors, especially those that are not abundant in the cell. The mouse ODC mRNA contains a long 5’ untranslated region
Figure 1.4. Transcriptional regulation of ODC by c-Myc.

The CACGTG c-Myc consensus sequence is the binding site for both the c-Myc:Max activating complex and the inactivating Mnt:Max complex. In response to stimuli such as mitogens, c-Myc is activated and the c-Myc:Max complex displaces the Mnt:Max complex by binding to the CACGTG motif. The c-Myc:Max complex stimulates ODC transcription causing an increase in cell proliferation, transformation potential, and apoptosis.

Source: (Nilsson, Maclean et al. 2004).
(UTR) that is approximately 300 bases in length (Gupta and Coffino 1985; Kahana and Nathans 1985; Coffino and Chen 1988). The size of this region, in conjunction with a high G-C content on the 5’ distal end, promotes the formation of secondary structure on the ODC 5’UTR (Figure 1.5) (Gupta and Coffino 1985; Kahana and Nathans 1985; Pegg, Shantz et al. 1994). In addition, the 5’UTR contains a short internal open reading frame (ORF) that is located 150 bases upstream of the translational start site (Gupta and Coffino 1985; Kahana and Nathans 1985; Wen, Huang et al. 1989). These features inhibit ODC translation (Van Steeg, Van Oostrom et al. 1991; Shantz and Pegg 1999). The effect of the ODC 5’UTR on translation has been investigated by many groups. Manzella et al. found, using reporter assays, that the rat 5’UTR of ODC inhibited translation. In addition, they showed that the 130 base G-C rich region was a key component in the translational repression of the 5’UTR. The 3’UTR decreased the inhibitory effects of the 5’UTR on ODC translation, although the mechanism of why this occurs remains unanswered (Manzella and Blackshear 1990).

There is a vast body of literature describing ODC translation in response to activated Ras (Holtta, Sistonen et al. 1988; Shantz 2004; Origanti and Shantz 2007). Ras is an essential player in the receptor mediated signal transduction pathway that regulates growth and differentiation. Constitutively overexpressing Ras has been linked to cancer [Reviewed in (Downward 2003)]. One study demonstrated that overexpression of a mutated and activated form of Ras, denoted as Ras12V, in NIH-3T3 cells resulted in a 20-fold induction of ODC enzyme activity. The increase in ODC enzyme activity was not due to a profound increase in transcription or RNA stability, but rather, was due to increased translation efficiency of the ODC mRNA (Shantz 2004). Another study showed a significant induction of ODC enzyme activity when rat intestinal epithelial (RIE-1) cells constitutively overexpressed Ras12V. An increase in cap-independent internal ribosomal entry site (IRES)-mediated translation was observed in
The 5'UTR sequence begins at nucleotide -313 and ends at nucleotide -1. Thus, the length of the mouse ODC 3'UTR is approximately 300 bases. An internal open reading frame begins at nucleotide -157. The length, as well as a high G-C content, promotes the formation of secondary structures. The ΔG of the structure was predicted to be -115 kcal/mol.

Source: (Shantz and Pegg 1999).
these cells and this increase in translation was mediated through the Raf/MEK/ERK and PI3-kinase pathways (Origanti and Shantz 2007).

Cap-dependent translation of ODC has been extensively studied. Studies have shown that overexpression of the translation factor eIF4E in NIH-3T3 cells, deemed 4EP2 cells, increases ODC enzyme activity through 5'UTR mediated protein synthesis (Shantz and Pegg 1994; Shantz, Hu et al. 1996). In a study using AR4-2J pancreatic tumor cells, increased phosphorylation of the eIF4E regulatory protein, 4E-BP1, induced ODC translation initiation (Pyronnet, Gingras et al. 1998). Similarly, in rat intestinal (IEC-6) cells Rapamycin, which blocks the phosphorylation of 4E-BP1, also inhibited ODC induction during serum stimulation (Seidel and Ragan 1997). These studies demonstrate the importance of the translation factor eIF4E for efficient ODC translation. Figure 1.6 reiterates some of the examples of ODC transcriptional and translational regulators, and emphasizes the importance of activated Ras and its downstream targets in ODC transcription and translation.

1.4.4 Degradation of ODC

The ODC protein is rapidly turned over, with a half-life ranging from 10 to 60 minutes [Reviewed in (Wallace, Fraser et al. 2003)]. The degradation process for ODC is unique in that it is ubiquitin independent (Murakami, Matsufuji et al. 1992). Alternatively, ODC non-covalently associates with a protein denoted as antizyme (AZ) which directs the protein to the 26 S proteasome for degradation [Reviewed in (Pegg 2006)]. ODC proteasomal degradation begins at the COOH terminus. Deletion of the 37 amino acid residues which comprise the COOH terminus makes the ODC protein more stable in the presence of AZ. Furthermore, the attachment of these residues to classically stable proteins causes them to become unstable (Zhang, Pickart et al. 2003; Zhang, MacDonald et al. 2004).
Figure 1.6. The Ras pathway activates both the transcription of the ODC gene and translation of ODC mRNA.

ODC transcription is mediated through c-Myc as described in Section 1.4.2. ODC translation is mediated through eIF4E as described in section 1.4.3. See text for details.

Modified from: (Shantz 2007).
The AZ protein was first discovered in 1976 by Canellakis who named it ODC antizyme because its synthesis was induced by the product of the enzyme (Heller, Fong et al. 1976). Antizyme is a family of three proteins that are differentially distributed; however, all antizymes function to inhibit ODC enzyme activity (Ivanov, Gesteland et al. 1998; Ivanov, Rohrwasser et al. 2000; Pegg 2006). The best characterized, and the one which will be discussed herein, is AZ1.

AZ1 is synthesized in a polyamine-dependent manner, with high polyamine content inducing AZ1 synthesis (Nilsson, Koskiniemi et al. 1997). The translation of AZ1 is regulated by a frameshift event (Figure 1.7). Briefly, the AZ1 RNA message consists of two overlapping ORFs, a short ORF1 and longer ORF2. ORF2 is in the +1 frame in relation to ORF1, and ORF2 does not contain its own initiation codon. Thus, the translation of ORF2 depends on the failure of the ribosome to terminate at the end of ORF1 and move to the +1 open reading frame of ORF2. The frameshifting event necessary for this is dependent on polyamine content. However, the exact mechanism behind this phenomenon remains elusive. In addition to polyamines regulating AZ1 translation, they also regulate AZ1 degradation and transcription. AZ1 is degraded in an ubiquitin-dependent manner and this is inhibited by excessive polyamine content in the cell [Reviewed in (Pegg 2006)].

AZ1 binds to the ODC monomer, thus creating a heterodimer that has no enzymatic activity, and directs it to the 26 S proteasome for degradation. Once ODC is degraded, the AZ1 protein can be recycled. The interaction site between ODC and AZ1 is located between residues 117-140 on the ODC protein, and between residues 106-212 on the AZ1 protein (Li and Coffino 1992; Li and Coffino 1994; Hoffman, Carroll et al. 2005).
AZ1 is synthesized in a polyamine-dependent manner. The translation of AZ1 is regulated by a frameshifting event in which the failure of the ribosome to terminate at the end of ORF1 allows the ribosome to continue to translate ORF2. AZ1 protein binds to the ODC monomer creating a heterodimer that has no enzymatic activity. AZ1 thus directs ODC to the 26S proteasome for degradation, and is recycled for future use.

Modified from: (Pegg 2006).
The importance of ODC is emphasized by the intricate management of ODC enzyme activity through transcription, translation, protein degradation, and, as described in the studies presented in this thesis, mRNA stability.

1.5 ODC and cancer

1.5.1 Overview

ODC enzyme activity has been shown to be elevated in numerous types of epithelial cancers including breast, colon, prostate, and skin [Reviewed in (Gerner and Meyskens 2004; Gilmour 2007)]. In addition, increased ODC enzyme activity has been shown to transform cell lines in vitro (Auvinen, Paasinen et al. 1992; Moshier, Dosescu et al. 1993). Particularly in the skin, the link between elevated ODC enzyme activity and skin tumor formation is indisputable, thereby making ODC an attractive chemoprevention target for combating skin cancer.

The enzyme ODC is necessary for normal cell growth and development [Reviewed in (Pegg 1988)]. Upon its discovery, ODC was associated with cancer, specifically sarcomas and hepatomas (Russell and Snyder 1968). Since then, ODC induction, with subsequent increases in putrescine, has been linked to numerous epithelial cancers, including NMSC (O'Brien, Simsiman et al. 1975; O'Brien, Simsiman et al. 1975; Gerner and Meyskens 2004; Casero and Marton 2007; Gilmour 2007).

1.5.2 Colon cancer

Recently, a correlation between a single-nucleotide polymorphism (SNP) in the Odc promoter and colon cancer risk was assessed. These studies revealed that those taking aspirin who were homozygous G at the (G315A) SNP site or heterozygous G/A at the site, were more likely to develop colon polyps than homozygous A individuals taking aspirin. The A-allele was also shown to reduce the risk of colon cancer. This was because the c-Myc antagonist MAD1 was shown to selectively repress Odc expression.
in an A-allele specific manner. This study demonstrated a correlation between increased ODC enzyme activity and colon cancer (Martinez, O’Brien et al. 2003). Additionally, it has been shown that human colonic adenocarcinomas displayed 6.5 times higher ODC enzyme activity than non-transformed tissue (Rozhin, Wilson et al. 1984).

1.5.3 Breast cancer

ODC induction and increased levels of polyamines have also been associated with breast cancer (Manni, Grove et al. 1995; Manni, Mauger et al. 1996). A positive correlation between elevated polyamine content and tumor grade has been exhibited (Kingsnorth, Wallace et al. 1984). To further demonstrate that ODC is necessary for breast tumor development, the pharmacological inhibitor of ODC, α-difluoromethylornithine (DFMO), was given to 1-methyl-1-nitrosourea (MNU) initiated rats (1% w/v in drinking water). DFMO-treated rats displayed a mammary tumor incidence rate of 59% compared to a 100% tumor incidence rate in non-DFMO treated littermates illustrating a positive correlation between ODC enzyme activity and mammary tumor formation (Thompson, Herbst et al. 1984).

1.5.4 Prostate Cancer

Increased levels of ODC enzyme activity have been shown in cases of prostate cancer. The transgenic adenocarcinoma mouse prostate (TRAMP) model for prostate cancer has been used to show a correlation between the progression of the disease and ODC enzyme activity. TRAMP mice are prone to prostate cancer with 100% of the males displaying adenocarcinomas. Treatment with DFMO resulted in a significant decrease in prostate weight and overall metastasis in these mice, demonstrating a link between high ODC enzyme activity and prostate cancer development (Gupta, Ahmad et al. 2000).
1.5.5 Non-melanoma skin cancer

ODC and its role in skin carcinogenesis have been studied for over thirty years. Studies utilizing either the multi-stage chemical carcinogenesis model or UV irradiation demonstrate that upregulation of ODC is both necessary and sufficient for the onset of skin tumors (O'Brien, Simsiman et al. 1975; Rosen, Gajic et al. 1990; Ahmad, Gilliam et al. 2001). Mice that were treated topically with TPA displayed increased ODC enzyme activity five hours after application of the tumor promoting agent (O'Brien 1976). In C57BL/6J transgenic mice that overexpress ODC in the skin via the Keratin 6 promoter, treatment with a tumor promoting agent was not necessary for the onset of skin tumors after initiation with DMBA, thus suggesting that ODC overexpression is sufficient for tumor promotion in the mouse skin cancer model (O'Brien, Megosh et al. 1997). Treating ODC overexpressing mice with DFMO blocked the formation of tumors in initiated mice. Furthermore, treating tumor bearing mice with DFMO led to tumor regression (Peralta Soler, Gilliard et al. 1998). Smith et al. demonstrated that mice overexpressing both the Ha-ras gene and ODC developed spontaneous tumors without the application of either a carcinogen or tumor promoting agent. A high percentage of these tumors progressed to form carcinomas. The formation of tumors was ODC dependent, as treatment with DFMO ablated tumor formation (Smith, Trempus et al. 1998). These results suggest that ODC and Ha-ras overexpression are sufficient to cause skin neoplasia in vivo. Furthermore, overexpression of the endogenous inhibitor of ODC, AZ1, greatly reduces tumor incidence and multiplicity in mice treated with the DMBA and TPA illustrating that ODC plays a causal role in skin tumorigenesis (Feith, Shantz et al. 2001).
1.6 Post-transcriptional regulation

1.6.1 Overview

Post-transcriptional regulation is a rapid mechanism of controlling gene expression. It is known that messenger RNA (mRNA) is transcribed in the nucleus where it is processed via splicing and the additions of the poly(A) tail and 5' 7-methylguanosine cap (5' cap). Once fully processed, the mRNA is able to shuttle out of the nucleus and into the cytoplasm where it can undergo various fates—it can be translated into protein, it can be degraded, or it can bind to an RNA binding protein (RBP). The RBP can influence mRNA degradation, affect translation efficiency, or shuttle the transcript to a processing body (P-body) for storage (Figure 1.8) [Reviewed in (Shyu, Wilkinson et al. 2008)].

1.6.2 Translation

Translation is the action by which an mRNA transcript is utilized to produce a protein product. Translation is comprised of three steps: initiation, elongation, and termination. The process of translation is illustrated in Figure 1.9. The initiation step of translation is rate-limiting, and thus is the most common target of translational control. The 40S ribosome complex, along with other initiation factors, creates the 43S pre-initiation ribosomal complex that binds to the mRNA. This 43S complex consists of eIF1, eIF1A, eIF3, and eIF5, as well as a ternary complex which consists of a methionine-loaded initiator tRNA that recognizes the AUG translation start site and eIF2 coupled to GTP.

During translation initiation, the 5' cap of the mRNA is bound by eIF4F, a heterotrimeric protein complex consisting of the backbone, eIF4G, eIF4E, and the RNA helicase eIF4A. It is eIF4E which directly binds to the 5' cap. In addition, eIF4G interacts with the poly(A) tail binding protein (PABP). The interactions between eIF4G and PABP lead to the circularization of the mRNA, which brings the 5' and 3' ends closer
Figure 1.8. Post-transcriptional regulation of labile mRNAs.

Mature RNAs shuttle into the cytoplasm where they can undergo various fates. They can be degraded, they can be translated into protein, or an RNA binding protein can bind to the 3’UTR at adenosine- and uracil-rich elements (AREs). Once bound, the RBP can shuttle the mRNA to a processing body (P-body) for storage, can influence the efficiency of translation, or can influence the degradation of the bound mRNA transcript. Upon leaving the P-body, the mRNA can be either translated or degraded.
Figure 1.9.  Schematic of mRNA translation.

Refer to section 1.6.2 for details.

Source: (Gebauer and Hentze 2004).
together. The RNA helicase, elf4A, aids in both the binding of the ribosome to the mRNA and its movement down the 5' UTR. Once the elf4F cap complex is bound, the 43S ribosome complex is recruited, through the action of elf4G. This recruitment involves the bridging of elf3 and elf4F. The 43S initiation complex moves down the 5' UTR scanning in the 5' to 3' direction in search of an AUG start codon that is surrounded by a Kozak sequence, a sequence in which a purine (adenosine or guanosine) is three bases upstream of the AUG start site and G at the +4 site. This sequence thus directs the translation of the mRNA. Once a start codon is found, the 48S initiation complex is formed, and hydrolysis of GTP by elf2 releases the Met-tRNA into the P site of the 40S ribosome subunit and then elf2 dissociates from the complex. During this step elfs 1, 1A, 3, and 5 also dissociate. Elf5B-GTP binds to the complex and the large 60S ribosomal subunit joins the 40S Met-tRNA-mRNA complex to create the 80S initiation complex. This then triggers the hydrolysis of GTP by elf5B which causes the dissociation of elf5B and GDP. One factor which regulates translation is the availability of elf4E for binding to elf4G. In most eukaryotic cells, elf4E has a natural inhibitor, 4E-BP1. Hypophosphorylated 4E-BP1 competes with elf4G for elf4E binding. Upon phosphorylation of 4E-BP1, the interaction between 4E-BP1 ad elf4E becomes weak, thereby releasing elf4E from 4E-BP1 and allowing it to form the cap complex for translation [Reviewed in (Gingras, Raught et al. 1999; Gebauer and Hentze 2004; Kapp and Lorsch 2004)].

1.6.3 Messenger RNA degradation

Degradation of mRNA is a process orchestrated by both cis- and trans-acting factors. RNA components that regulate decay include: the 5'cap, 5'UTR, protein coding region, 3'UTR, and poly(A) tail. The 5' cap in mammals protects the body of the mRNA from exonucleases while at the same time enhancing translation initiation. Similarly, the
3’ poly(A) tail, when intact, keeps the body of the mRNA safe from degradation by exonucleases.

Degradation of an mRNA usually begins with the deadenylation of the poly(A) tail through the CCR4-NOT complex, PARN, or the PAN2-PAN3 complex. After deadenylation, the mRNA can be degraded through 2 processes: 5’→3’ or 3’→5’ exonucleolytic decay [Reviewed in (Parker and Song 2004; Garneau, Wilusz et al. 2007)]. During 5’→3’ exonucleolytic decay, the 5’ cap is removed, and the body of the mRNA undergoes decay mediated by XRN1. Decapping occurs through a dimer DCP1 and DCP2, and the bridging component Ge-1. Additionally, accessory factors are required for decapping such as the Lsm1-7 complex as well as RAP55. Hydrolysis of the cap by the DCP complex releases m^7GDP, leaving the mRNA with a 5’ monophosphate which is the preferred substrate for XRN1 [Reviewed in (Eulalio, Behm-Ansmant et al. 2007; Franks and Lykke-Andersen 2008)]. In contrast, once deadenylation has occurred, the mRNA can be degraded in a 3’→5’ mechanism. The exosome and SKI complex, a 10-12 subunit complex made of 6 proteins is responsible for this degradation pathway. Furthermore, mRNA can be internally cleaved by an endonuclease in a deadenylation-independent manner. The two resulting fragments are subsequently degraded. The 5’ fragment is degraded by exosomes, while the 3’ fragment is degraded by XRN1 (Figure 1.10) [Reviewed in (Guhaniyogi 2001; Garneau, Wilusz et al. 2007)].

1.6.4 Processing body (P-body) storage

P-bodies are aggregates of translationally repressed mRNAs that are associated with translation or mRNA decay machinery. These structures were first described by Bashikorov et al. in 1997 after the discovery that the XRN1 protein localized to granular foci within the cytoplasm of mouse cells (Bashkirov, Scherthan et al. 1997). To date, the exact protein composition of P-bodies is not known. However, a conserved core of
mRNA are degraded three ways: 5'→3' exonucleolytic decay, 3'→5' exonucleolytic decay, and by deadenylation-independent mechanisms. Both 5'→3' and 3'→5' exonucleolytic decay require deadenylation. During deadenylation-independent mRNA decay, an endonuclease cleaves the mRNA and the resulting fragments are degraded in a 5'→3' or 3'→5' fashion. Refer to Section 1.6.3 for more details.

Modified from (Eulalio, Behm-Ansman et al. 2007).
proteins are found in P-bodies in both yeast and mammals and include: DCP1, DCP2, Lsm1-7 complex, XRN1, the CCR4-NOT complex, the RNA binding protein TTP, components of the RNAi silencing complex, and the translation initiating factor eIF4E [Reviewed in (Anderson and Kedersha 2006; Parker and Sheth 2007)].

The mechanism of P-body assembly is not fully understood. P-bodies assemble when the 5’→3’ decay machinery is overloaded with mRNA substrate or when the mRNA decay pathway is impaired. Conversely, P-bodies decrease in both size and number when the cellular mRNA level is reduced. P-bodies are reduced when transcription is blocked or when mRNAs are trapped in association with ribosomes without elongation (via Cycloheximide treatment) [Reviewed in (Parker and Sheth 2007; Franks and Lykke-Andersen 2008)]. In one study using cells in which decapping was blocked, it was shown that inhibition of polysome disassembly by Cycloheximide resulted in P-body loss (Brengues, Teixeira et al. 2005). Furthermore, P-body size has been linked to cell cycle progression, with P-bodies becoming larger during late S and G2 phases (Yang, Jakymiw et al. 2004). Hence, P-bodies are quite dynamic and are influenced by intracellular conditions such as mRNA accumulation and translation. In fact, P-bodies require mRNA for their formation because treatment of cells with RNase A disrupts P-body formation and size (Teixeira, Sheth et al. 2005). P-bodies are defined as the site of mRNA decay because mRNA decay machinery is localized to the P-bodies. However, recently it has been shown that intact mRNAs can be released from P-bodies into the cytoplasm, where they can be translated into protein, designating P-bodies as not only a site for decay but also a site for mRNA storage and sequestration (Brengues, Teixeira et al. 2005; Garneau, Wilusz et al. 2007; Parker and Sheth 2007). Furthermore, P-bodies have recently been shown as sites of microRNA (miR)-mediated translational silencing. Reporter constructs targeted by miRs have been shown to accumulate in P-bodies (Liu, Valencia-Sanchez et al. 2005). These data further expand
the link between P-bodies and mRNA decay. The field of P-body research is still in its infancy; therefore, there is still much to be learned about these macromolecules including: how they are formed and regulated, what proteins localize to the P-bodies, and their precise role in normal cell physiology.

### 1.7 RNA binding proteins (RBPs)

#### 1.7.1 Overview

RBPs are able to bind to labile mRNA transcripts at adenosine- and uracil-rich elements (AREs) and either increase or decrease both mRNA stability and translational efficiency. Numerous RBPs have been documented with some of the best studied being the stabilizing RBP human antigen R (HuR) and the destabilizing RBP tristetraprolin (TTP). Such RBPs are important because it is predicted that there are 4000 ARE containing mRNAs (Bakheet, Williams et al. 2006). Given that approximately 5% of all human mRNAs contain AREs, it is both relevant and necessary to understand the mechanism of RBP regulation and RBP interactions with mRNA transcripts (Bakheet, Williams et al. 2006).

#### 1.7.2 Human antigen R (HuR)

##### 1.7.2.1 Overview

Hu proteins were originally identified as tumor antigens in non-small lung cancers associated with paraneoplastic neurological disorder (Dalmau, Fumeaux et al. 1990). Due to the high homology between the embryonic lethal abnormal vision (ELAV) family of RNA binding proteins discovered in *Drosophila melanogaster* and Hu proteins, Hu proteins are often referred to as members of the ELAV family. Elav11 is essential for neuronal development in drosophila (Campos, Grossman et al. 1985). Similarly, HuR’s family members HuB, HuC, and HuD, are solely expressed in neuronal cells. However, HuR is ubiquitously expressed (Ma, Cheng et al. 1996).
HuR was originally cloned in *Xenopus laevis* and conserved homologues were identified in both the mouse and human (Good 1995; Ma, Cheng et al. 1996; Keene 1999). The *HuR* gene is located on 19p13.2 in humans, and on chromosome 8 in the mouse genome (Ma and Furneaux 1997).

1.7.2.2 Regulation

The HuR promoter extensively regulates HuR transcription. It is approximately 500 bases long and contains numerous initiation sites as well as various transcription factor binding sites (King, Fuller et al. 2000). For example, it has been suggested that NF-KB is a transcription factor for HuR (Kang, Ryu et al. 2008). Although the first 130 nucleotides of the HuR promoter are GC-rich, no CpG island methylation has been recorded within this region (King, Fuller et al. 2000; Kang, Ryu et al. 2008).

As HuR is predominately located in the nucleus, the change in HuR cellular distribution between the cytoplasm and nucleus is recognized as the main mechanism by which HuR influences mRNA transcript stability [Reviewed in (Doller, Pfeilschifter et al. 2008)]. To date, only a few post-translational modifications have been recorded which alter the localization of HuR. PKCα has been shown to phosphorylate HuR at S158 and S221. The phosphorylation of HuR at these residues causes the accumulation of cytoplasmic HuR and thereby increases the stability of the COX-2 mRNA. Overexpression of PKCα led to increased cytoplasmic HuR suggesting that PKCα plays a role in the cytoplasmic accumulation of HuR (Doller, Huwiler et al. 2007). Similarly, PKCδ phosphorylates HuR at S221 and S318 and causes the cytoplasmic accumulation of HuR. This phosphorylation of HuR can also increase the binding affinity between HuR and its target mRNA (Doller, Akool el et al. 2008). The importance of S221 phosphorylation was further exhibited in a set of experiments in which mutating S221 to A221, a non-phosphorylatable mutant, caused HuR sequestration in the nucleus after treatment with angiotensin II (AngII), a known inducer of HuR cytoplasmic shuttling.
Additionally, Cyclin-dependent Kinase 1 (Cdk1) phosphorylates HuR at S202, which leads to the nuclear accumulation of HuR mediated through the 14-3-3 protein chaperone (Figure 11.1B) (Kim, Abdelmohsen et al. 2008). Furthermore, the Gorospe group created an HuR mutant, S242A, which was shown to accumulate in the cytoplasm and bind to target mRNAs. In contrast, overexpression of the phosphomimetic mutant S242D led to the nuclear accumulation of the exogenous HuR. The putative kinase for S242 remains elusive as do other kinases which phosphorylate HuR and cause alterations to its localization (Kim, Yang et al. 2008).

In addition to known modifications to the HuR protein by PKC-mediated pathways, other pathways such as AMP-activated Protein Kinase (AMPK) have been described to regulate HuR localization. Some studies have shown that activation of AMPK correlates with a decreased cytoplasmic accumulation of HuR (Wang, Fan et al. 2002). This regulation of HuR localization is mediated through binding with importin α1, an adaptor protein involved in nuclear import. It has been suggested that activated AMPK regulates the direct phosphorylation and indirect acetylation (possibly through the AMPK-mediated phosphorylation of p300 which in turn acetylates importin α1) of importin α1 thereby causing importin α1 and the bound HuR to re-locate to the nucleus (Wang, Yang et al. 2004). Moreover, the nuclear export of HuR is mediated by its association with transportin 1 (TRN1) and TRN2 and the nuclear ligands pp32 and APRIL. These contain nuclear export signals that are recognized by CRM1, and suggest that CRM1 is another upstream cascade involved in HuR localization (Gallouzi and Steitz 2001; Doller, Pfeilschifter et al. 2008).

1.7.2.3 Structure

The HuR protein contains 3 RNA recognition motifs (RRMs) (Ma, Cheng et al. 1996). The first two RRM bind ARE-containing mRNA. A hinge region is downstream of the first two RRM and contains the HuR nucleocytoplasmic shuttling sequence.
Figure 1.11. Schematic of the HuR protein and post-translational modification sites within the HuR protein.

A. The HuR protein consists of 3 RNA recognition motifs (RRMs). RRM1 and 2 lie in tandem, while RRM3 is separated by RRM1 and 2 by the hinge region of the HuR protein. The hinge region contains the HuR nucleocytoplasmic shuttling sequence (HNS) which is located between residues 205 and 237. RRM1 and 2 have been shown to bind to the mRNA while RRM3 has been shown to bind to the poly(A) tail of the mRNA. B. The HuR protein is post-translationally modified by numerous proteins. The post-translational modifications are highlighted: gray: PKCα phosphorylation sites, red: PKCδ phosphorylation sites, blue: Cdk-1 phosphorylation site.

Modified from: (Doller, Pfeilschifter et al. 2008).
(HNS), spanning residues 205-237, and containing both nuclear import and export signals (Fan and Steitz 1998). RRM3, which is downstream of the hinge region, binds the poly (A) tail of the mRNA as well as maintains the stability of the RNA-protein complex (Figure 1.11A) (Ma, Chung et al. 1997; Fan and Steitz 1998). Recently, the first HuR crystal structure has been solved for RRM1 and shows that RRM1 consists of 2 alpha-helices and 4-stranded beta-sheets (Benoit, Meisner et al. 2010).

1.7.2.4 Function

Cytoplasmic HuR was first shown to bind to and stabilize mRNA transcripts in 1998 when two groups demonstrated HuR binding to c-fos and beta globin reporter mRNAs containing AREs (Fan and Steitz 1998; Peng, Chen et al. 1998). Since then, HuR has been shown to influence the stability of numerous labile mRNA transcripts. Such target transcripts impact proliferation, angiogenesis, immune response, and cell survival [Reviewed in (Hinman and Lou 2008)].

In addition to stabilizing labile mRNA transcripts, HuR has also been shown to influence mRNA translation by either increasing or decreasing translational efficiency. For example, HuR has been shown to not only increase the RNA stability but also enhance the translational efficiency of various transcripts, including MAP Kinase Phosphatase 1 (MKP-1), thrombospondin-1 (TSP1), and p53 (Kuwano, Kim et al. 2008; Mazan-Mamczarz, Hagner et al. 2008; Tong and Pelling 2009). However, it has also been shown that HuR decreases translation efficiency (Meng, King et al. 2005).

1.7.2.5 Transgenic mouse models for studying HuR

HuR knockout is lethal by day E14.5; thus, an HuR knockout mouse is unavailable. However, HuR knockout mouse embryonic fibroblasts (MEFs) are available from E12.5 mice (Katsanou, Milatos et al. 2009). Conditional HuR knockout mice, using a floxed HuR allele, have been created (Katsanou, Milatos et al. 2009; Papadaki, Milatos et al. 2009). The use of these conditional HuR knockout mice is recent but promising
because this technology can be used to create more cell type specific HuR knockout mice in order to solidify the roles HuR plays in development.

There has also been success in creating HuR overexpressing transgenic mice. For example, a transgenic mouse overexpressing HuR in the myeloid lineage through a lysozyme promoter was regulated through the use of the tetracycline analog, Doxycycline (Dox). These mice were viable and overexpressed the HA-tagged HuR. The studies revealed that overexpression of HuR inhibited the inflammatory response in these animals (Katsanou, Papadaki et al. 2005).

Studies using the transgenic HuR mice briefly discussed above reveal the importance of HuR in not only immunity, but also in embryonic development. Currently, there are no published data using these mouse models to study carcinogenesis. However, HuR mouse models can provide a powerful tool to elucidate the role HuR plays in tumorigenesis in vivo.

1.7.3 Tristetraprolin (TTP)

1.7.3.1 Overview

TTP is a member of a family of RNA binding proteins which contain Cys-Cys-Cys-His-zinc (CCCH-zinc) finger RNA-binding domains, and is located at 19q13.1 in the human genome and on mouse chromosome 7 [Reviewed in (Sandler and Stoecklin 2008)]. This protein was originally described in 1990 as a gene rapidly induced by insulin in a differential hybridization screen. It was shown that TTP mRNA was undetectable in unstimulated HIR 3.5 cells, NIH-3T3 cells which overexpress the human insulin receptor, but was rapidly induced 10 minutes after insulin stimulation. Upon further investigation and sequencing of this unknown protein, it was discovered that the sequence was rich in proline and serine residues, containing 3 blocks of 4 proline residues (SSPPPPG). Based on this observation, the unknown protein was named TTP for tristetraprolin (Lai, Stumpo et al. 1990). Concomitantly, TTP was partially cloned as a
gene that was responsive to treatment with TPA; the unknown gene was named TPA-induced sequence 11 (TIS11) (Varnum, Lim et al. 1989). A third laboratory also cloned TTP as a gene that was induced by serum naming it Nup475 (DuBois, McLane et al. 1990).

1.7.3.2 Regulation

The promoter region of TTP was investigated due to the rapid accumulation of message after treatment with inducing agents such as insulin and TPA. The promoter contains transcription factor binding sites on the proximal region. For example, the promoter contains both an AP-2 site and an early growth receptor gene product-1 (Egr-1) site (DuBois, McLane et al. 1990; Lai, Thompson et al. 1995). These sites are necessary for TTP inducibility by insulin and serum (Lai, Thompson et al. 1995).

Like HuR, TTP is post-translationally modified, and a few upstream targets have been identified. The p-38-MAPK pathway has been implicated in the phosphorylation of TTP at serines 52 and 178. Specifically, a downstream target of the MAPK pathway, MAPKAP Kinase 2 (MK2), has been shown to phosphorylate TTP at these residues (Chrestensen, Schroeder et al. 2004). This phosphorylation permits the binding of TTP to the 14-3-3 adaptor protein thereby causing a decrease in TTP’s ability to destabilize mRNA transcripts. However, MK2 activity is balanced by protein phosphatase 2A (PP2A), which competes with the 14-3-3 protein for TTP binding (Sun, Stoecklin et al. 2007; Sandler and Stoecklin 2008). When bound, PP2A dephosphorylates TTP, which subsequently activates mRNA decay (Figure 1.12).

1.7.3.3 Function

TTP is known to destabilize mRNA transcripts, thereby promoting mRNA decay. It has been estimated that TTP binds to over 250 transcripts, although to date, 250 TTP-mRNA interactions have not been described (Lai, Parker et al. 2006). Among its targets, TTP has been shown to destabilize tumor necrosis factor α (TNF-α), granulocyte
Figure 1.12. TTP is regulated by MK2 phosphorylation and PP2A dephosphorylation.

The p-38-MAPK pathway activates MK2 which phosphorylates TTP at serines 52 and 178. This phosphorylation induces binding of TTP to the 14-3-3 adaptor protein thereby decreasing TTP’s ability to destabilize labile mRNA. PP2A, a phosphatase, competes with the 14-3-3 protein for binding to TTP. Once bound, PP2A dephosphorylates TTP, which activates TTP and mRNA decay.

Source: (Sandler and Stoecklin 2008).
macrophage-colony-stimulating factor (GM-CSF), and interleukin 3 (IL-3) (Lai, Carballo et al. 1999; Carballo, Lai et al. 2000; Stoecklin, Ming et al. 2000). In addition to destabilizing mRNA transcripts, TTP has also been implicated in the promotion of ARE mediated decay. The TTP protein has been found to interact with components of the RNA decay machinery, specifically DCP1, DCP2, and XRN1 (Fenger-Gron, Fillman et al. 2005; Lykke-Andersen and Wagner 2005). Moreover, TTP has been shown to interact with P-body proteins, namely CCR4 (Lykke-Andersen and Wagner 2005). To further establish the link between TTP, P-bodies, and mRNA decay, it has been demonstrated that tethering an ARE-less mRNA to TTP results in the mRNA localizing to the P-bodies and degradation of the mRNA (Franks and Lykke-Andersen 2007).

1.7.3.4 TTP knockout mouse model

The TTP knockout mouse was created by placing the neomycin resistance gene in the protein coding region of the second exon of TTP, thereby creating multiple stop codon sites which were located upstream of its two zinc fingers. This insertion renders the TTP protein nonfunctional. TTP knockout mice are viable, and Northern blot analysis demonstrated ablation of the TTP message in numerous tissues of the knockout mice. Furthermore, fibroblasts from the knockout mice exhibited no TTP. TTP knockout mice are phenotypically normal at birth; however, between the ages of 1 and 8 weeks, these mice exhibit cachexia, dermatitis, conjunctivitis, and arthritis. Moreover, these mice have life-spans of 7 to 16 months. It appears that the main mechanism of this phenotype is due to increased TNF-α in these mice, as injection of knockout mice with a TNF-α antibody reverted the knockout phenotype to a wild-type phenotype (Taylor, Carballo et al. 1996). These mice, and the subsequent MEFs that are obtained from TTP knockout mice, provide a powerful tool for studying the role TTP plays in mRNA stability and decay.
1.8 RNA binding proteins and cancer

The correlation between the localization and/or accumulation of the RBPs HuR and TTP and cancer has been well documented. Recent results have shown in numerous cancers that HuR, particularly cytoplasmic HuR, is elevated (Hasegawa, Kakuguchi et al. 2009). Often, the accumulation of cytoplasmic HuR is associated with poor disease prognosis (Lopez de Silanes, Fan et al. 2003; Denkert, Weichert et al. 2004; Heinonen, Bono et al. 2005; Koljonen, Bohling et al. 2008).

As previously mentioned, HuR was originally discovered as a tumor antigen present in individuals with non-small cell lung carcinomas (Dalmau, Furneaux et al. 1990). A survey conducted on healthy and cancerous tissues using human tissue arrays further linked HuR to cancer by showing that the expression of HuR was induced in most cancer tissues when compared to the normal tissue controls (Lopez de Silanes, Fan et al. 2003). Moreover, HuR was shown to accumulate in the cytoplasm of lung tumors (Blaxall, Dwyer-Nield et al. 2000), and 30 percent of women with invasive ductal breast carcinoma displayed increased cytoplasmic HuR levels. In a breast cancer study, cytoplasmic HuR presence was associated with high tumor grade (Heinonen, Bono et al. 2005). In patients with Merkel cell carcinoma, a form of skin cancer, it was shown that 30 percent of the primary tumors contained cytoplasmic HuR accumulation and that 60 percent of the lymph node metastases contained cytoplasmic HuR. The wild-type epidermis controls exhibited no cytoplasmic HuR. In contrast, normal epidermis and hair follicles exhibited nuclear accumulation of HuR, again linking HuR localization to cancer development (Koljonen, Bohling et al. 2008).

TTP has also been linked to cancer. A study in malignant glioma cells showed TTP to be highly expressed, but hyperphosphorylated. The group further showed that TTP and HuR competed for mRNA transcripts demonstrating a mechanism of competition between the two RBPs (Suswam, Li et al. 2008). TTP protein was shown to
be dramatically decreased in colon adenocarcinoma samples when compared to wild-type controls (Lee, Son et al. 2009). In invasive breast cancer cells, TTP was detected at lower levels when compared to control breast cell lines. Consequently, this decrease in TTP led to the induction of matrix metalloproteinase-1 (MMP-1), a gene known to be induced in breast cancer cell lines (Al-Souhibani, Al-Ahmadi et al. 2010). These and other observations strongly support the hypothesis that HuR and TTP, either alone or in concert with each other, play a role in cancer development.

The data described above demonstrate that ODC, HuR, and TTP act as causal factors in tumorigenesis. As ODC is intricately regulated, the mechanism of ODC mRNA stability control, particularly through the RBPs HuR and TTP, during skin neoplasia is in need of investigation. By understanding the interactions between ODC, HuR, and TTP, more potent chemoprevention and/or chemotherapeutic agents for NMSC can be explored.
Hypothesis and specific aims

**Hypothesis:** ODC enzyme activity and mRNA stability are induced in spindle cell carcinomas derived from mice that had been treated with DMBA-TPA when compared to non-transformed keratinocytes. We will test the hypothesis that the cytoplasmic accumulation and subsequent binding of the stabilizing RNA binding protein (RBP), HuR, to the ODC mRNA transcript accounts for the increased ODC stability and enzyme activity in skin tumor development.

**Specific Aim 1:** To characterize the phenotype of non-transformed and transformed keratinocytes by assessing the ODC enzyme activity, the ODC promoter strength, the ODC steady-state mRNA level, and the ODC mRNA stability in these cells.

**Specific Aim 2:** To evaluate what contribution the localization of the stabilizing RBP HuR has on ODC stability and ODC enzyme activity. Additionally, we will evaluate the contribution cytoplasmic HuR has on the mRNA stability of other transcripts implicated in carcinogenesis.

**Specific Aim 3:** To determine the effect the destabilizing RBP TTP has on ODC mRNA stability, ODC enzyme activity, and ODC 3’UTR mediated luciferase activity.

**Specific Aim 4:** To determine the influence the ODC 5’UTR and 3’UTR have on luciferase activity in normal keratinocytes and carcinoma-derived cell lines.
CHAPTER 2
Materials and Methods

2.1 Cell culture

The C5N and A5 mouse keratinocytes (a generous gift from Dr. Allan Balmain, UCSF, 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% penicillin streptomycin, and 1% glutamine (Invitrogen). These cells have been described previously (Zoumpourlis, Solakidi et al. 2003). Passages 5-20 were used in the experiments, and experimental results were consistent regardless of passage number. Stock flasks were incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂. Cells were passaged one time after thawing before use.

TTP⁺/⁺ and TTP⁻/⁻ mouse embryonic fibroblasts (MEFs, a generous gift from Dr. Perry J. Blackshear, NIEHS, Durham, NC) derived from E14.5 mouse embryos, were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 1% penicillin streptomycin, and 1% glutamine (Invitrogen). These cells have been described previously (Lai, Parker et al. 2006). Stock flasks were incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂. All cell morphology was assessed by light microscopy using the Nikon TMS (Nikon Instruments Inc., Melville, NY).

2.2 Western blot analysis

Western blotting was conducted as described previously (Origanti and Shantz 2007). Briefly, cells were extracted in 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 pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, 1
mM PMSF, 1X protease inhibitor cocktail] (Santa Cruz Biotechnology, Santa Cruz, CA). Total cellular protein (20 µg) was resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Pall Life Science, Pensacola, Florida). The antibodies recognizing HuR (Santa Cruz Biotechnology), GAPDH (BioDesign International, Saco, ME), Histone H3 (Cell Signalling), TTP (Santa Cruz), and α/β Tubulin (Cell Signalling) were all used at a 1:1000 dilution. The antibody recognizing Lamin A/C (a generous gift from Dr. Sergei Grigoryev, Pennsylvania State University College of Medicine, Hershey, PA) was diluted at a 1:2500 dilution.

2.3 Growth curve analysis

To assess the growth rates of the cells, both C5N and A5 cells were seeded at a density of 2000 cells per cm² on a 24 well plate. The cells were maintained in DMEM. The number of cells per cm² was measured at time 0 h (after allowing the cells to adhere for 16 h) and then measured daily until the cells reached the stationary phase. Cells were counted using a Coulter Counter (Coulter Electronics Inc., Hialeah, FL). Log of cells per cm² was plotted against time and the generation times were determined using exponential curve fit analysis.

2.4 ODC enzyme activity assay

C5N cells were seeded at a density of 150,000 cells per 6 cm plate while A5 cells were seeded at a density of 60,000 cells per 6 cm plate. Seventy-two h later, the cells had reached 70% confluency and were extracted in ODC harvest buffer [10 mM Tris-HCl pH 7.5, 2.5 mM dithiothreitol, and 0.1 mM EDTA]. ODC enzyme activity was determined by radiometric assay measuring the amount of ^14CO₂ released from each reaction in
which \[^{1}{^1}[^{14}\text{C}]-\text{ornithine}\] is enzymatically converted to \(^{14}\text{CO}_2\) by ornithine decarboxylase. Collection and analysis were carried out as previously described (Coleman 1998).

2.5 Polyamine content

Polyamine content was measured using reversed-phase HPLC analysis as described previously (Shantz, Holm et al. 1992). The data were normalized to total protein.

2.6 ODC promoter activity assay

The mouse ODC promoter region was cloned into the promoterless Basic-pGL3 vector (Promega, Madison, WI). This vector, herein denoted as pODCLuc, contained the Firefly luciferase gene downstream of the ODC promoter region. C5N and A5 cells were plated at a density of 200,000 cells per 6 cm plate. Twenty-four h later, each plate was either transfected with \(2\,\mu\text{g}\) of pODCLuc or a control vector, pGL3 Control, which contains a SV-promoter upstream of the Firefly luciferase reporter gene (Promega), using the transfection agent Lipofectamine 2000 as per manufacturer’s instructions (Invitrogen). In order to control for transfection efficiency, cells were transfected with pRL-SV40, a vector containing the Renilla luciferase reporter gene. The amount of pRL-SV40 used in the transfection was 10-fold less than the amount of either pODCLuc or pGL3 Control used (0.2 \(\mu\text{g}\) per plate). Forty-eight h after transfection, cells were extracted in 1X Passive Lysis Buffer (Promega) and assayed using the Dual-Luciferase Kit as per manufacturer’s instructions (Promega). The Firefly/Renilla ratio were recorded. The data were normalized to the pGL3 Control.
2.7 RNA extraction, Northern blot analysis, and Actinomycin D experiments

A5 and C5N cells were plated at a density of 150,000 and 185,000 cells per 6 cm plate respectively. Upon reaching 70% confluency, RNA was extracted using the TRIzol reagent according to the manufacturer’s instructions (Invitrogen). The RNA from two plates were combined to make each sample. For Northern blot analysis 2 µg or 20 µg of RNA was loaded onto a 1.2% (v/v) agarose-formaldehyde gel. The amount of total RNA loaded was dependent on the size of the gel running apparatus used. The RNA was subsequently transferred to a Hbond N+ membrane (GE, Piscataway, NJ). After transfer, the membrane was UV cross-linked using a Stratalinker (Stratagene, La Jolla, CA). Northern analysis was conducted using $^{32}$P labeled cDNA probes synthesized for ODC and Cyclophilin A. These probes were made using the Takara Ladderman Labeling Kit as per manufacturer’s instructions (Takara, Madison, WI). The primers were:

ODC

5’-CGAGAACCATGAGCAGCTTTAC-3’ (sense)

5’-GCATCCTTATCGTCAGAGGAAG-3’ (antisense)

Cyclophilin A

5’-GCAGGTCCATCTACGGAGAG-3’ (sense)

5’-CTGGGAACCGTTTGTGTTTGG-3’ (antisense)

The bands were quantitated using Syngene Software (Syngene, Frederick, MD) and the ODC mRNA data were normalized to that of Cyclophilin A. The stability of the ODC mRNA was assessed by the addition of Actinomycin D (5-10 µg), and RNA was extracted at 0 h, 4 h, and 8 h using the TRIzol reagent method. ODC mRNA half-life was determined using exponential curve fit analysis.
2.8 Quantitative Real-Time PCR analysis

Total RNA was isolated using the TRIzol reagent described above and used in reverse transcription and PCR amplification. cDNA was produced using the Transcriptor First Strand cDNA Synthesis Kit as per the manufacturer’s instructions (Roche, Nutley, NJ). qRT-PCR reactions were performed by the Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA), using the same primers for ODC and Cyclophilin A described above (Tm=58°C) and by using the Quantitect SYBR Green PCR Kit as per the manufacturer’s instructions (Qiagen, Valencia, CA). Depending on the conditions of the experiment, cDNA was either not diluted or diluted at 1:1 for sample measurements. For standard curve analysis, a combination of equal volumes of the experimental cDNA was diluted 1:3 and was used as the starting dilution. This sample was diluted 4 times thereafter to set up the dilution series. qRT-PCR was also used to assess HuR, COX-2, and VEGF content. The primers were:

HuR, Tm=53°C

5’-GGTTATGAAGACCACATGGC-3’ (sense)
5’-GAACAGACTTCGTAGTTCCTC-3’ (antisense)

COX-2, Tm=57°C

5’-AAGTCTAATGATCATATTTATTTATATGAAC-3’ (sense)
5’-CACAAGTATGACTCCTTCTCC-3’ (antisense)

VEGF, Tm=57°C

5’-TTTCAGTATTCTTGTTAATATTTATTTTC-3’ (sense)
5’-GTGACTGTCAAGGATCAGGGAG-3’ (antisense)

Analysis was performed by using the Step One Plus Real-Time PCR software (Applied Biosystems). The PCR conditions used were as follows: Denaturation 94°C-15s, Annealing-30s, Elongation 72°C-30s. The cycles were repeated 39 times. The data
were normalized to Cyclophilin A. When using qRT-PCR to determine mRNA half-life, exponential curve fit analysis was used.

2.9 Cellular fractionation

Cellular fractionation was accomplished through two different methods. The first method used the Pierce Ner-Per Nuclear and Cytoplasmic Fractionation Kit (Pierce, Rockford, IL). Fractionation using this kit was per the manufacturer's instructions. The second method involved the use of 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 at 1000 rpm for 5 min. The supernatant was extracted as the cytoplasmic lysate. Verification of the cellular fractionation experiments occurred via Western blot analysis using Histone H3, Lamin A/C, and α/β Tubulin antibodies to determine the purity of each fraction (Western blotting described in Section 2.2).

2.10 Preparation of synthetic RNA transcripts and biotin-labeled RNA-protein binding assays

2 µg cDNA from C5N and A5 cells was used as a template for PCR amplification of products containing the T7 promoter. cDNA was made as described in Section 2.8. The sense primers for each reaction contained the T7 RNA polymerase promoter sequence on the 5’ end and are listed below along with the antisense sequences for each T7 promoter containing PCR product. The T7 sequence is 5’-GCTTCTAATACGACTCACTATAGGGAGA-3’ and is denoted as (T7) in the primers below.
ODC 5'UTR, Tm=57ºC

(T7) 5'-GCCTCCCGGCGGAACCGATCG-3' (sense)
5'-GGTTCTCGATGTGCTTACAGGG-3' (antisense)

ODC coding region (CR), Tm=51.7ºC

(T7) 5'-ATGAGCAGCTTTACTAAGGACG-3' (sense)
5'-CTACACATTTGATCCTAGCAGA-3' (antisense)

ODC 3'UTR, Tm=51.7ºC

(T7) 5'-GGTTCTCGATGTGCTTACAGGG-3' (sense)
5'-TTGCTGTTGTTGAATTTATTAC-3' (antisense)

ODC 3'UTR F1, Tm=57ºC

(T7) 5'-ATGCCATTCTTGTAGCTTTC-3' (sense)
5'-CAGTAAGTGTCGACCCCATCT-3' (antisense)

ODC 3'UTR F2, Tm=57ºC

(T7) 5'-ATGCCATTCTTGTAGCTTTC-3' (sense)
5'-GCTACACATTTGATCCTAGCAGA-3' (antisense)

ODC 3'UTR F3, Tm=57ºC

(T7) 5'-CAGTAAGTGTCGACCCCATCT-3' (sense)
5'-AT GCCACATTTGATCCTAGCAGA-3' (antisense)

ODC 3'UTR F4, Tm=57ºC

(T7) 5'-ATGCCACATTTGATCCTAGCAGA-3' (sense)
5'-ATGCCACATTTGATCCTAGCAGA-3' (antisense)

ODC 3'UTR F5, Tm=50.2ºC

(T7) 5'-GTCTGTTGTTGAATTTATTAC-3' (sense)
5'-TTGCTGTTGTTGAATTTATTAC-3' (antisense)
ODC 3'UTR Deletion 1, Tm=57°C
(T7)5'-ATGCCATTCTTGATGCTCTTG-3' (sense)
5'-CATTCCCTGATGCCCCAGTTATTTCAAGATA-3' (antisense)

ODC 3'UTR Deletion 2, Tm=57°C
(T7)5'-ATGCCATTCTTGATGCTCTTG-3' (sense)
5'-CAAAGTTTCCATAGGAACACAGTAAGTG-3' (antisense)

ODC 3'UTR Deletion 3, Tm=56°C
(T7)5'-ATGCCATTCTTGATGCTCTTG-3' (sense)
5'-CCCAAATGCCTTAACACAAGCT-3' (antisense)

ODC 3'UTR Deletion 4, Tm=53°C
(T7)5'-CACACTTACTGTGTTCTATGGAACTTTT-3' (sense)
5'-TTGCTGTTGTTGAAATTTATTAC-3' (antisense)

ODC 3'UTR Deletion 5, Tm=50°C
(T7)5'-ATCCTTAAGAAGGCACCAAC-3' (sense)
5'-TTGCTGTTGTTGAAATTTATTAC-3' (antisense)

ODC 3'UTR (bases 1851-2151), Tm=56.3°C
(T7)5'-GGGACCATTCTTACTTACTGCTAG-3' (sense)
5'-TACTTGCAAACATTCCCTGATG-3' (antisense)

GAPDH, Tm= 62°C
(T7)5'-GAAACCCTGGACCACCCACCCCAG-3' (sense)
5'-GGGTGCAGCGAACTTTATTGATG-3' (antisense)

C-myc, Tm=50.2°C
(T7)5'-ACTGACCTAACTCGAGGAGGAG-3' (sense)
5'-GTATTTTTTCCAATTTTTAT-3' (antisense)
VEGF, Tm=55ºC

(T7)5'-TTTCAGTATTTGTTAATATTTAATTTTC-3' (sense)
5'-CTTTGAGATCGAATTTCTTTAATAC-3' (antisense)

COX-2, Tm=55ºC

(T7)5'-AAGTCTAATGATCATTTATTTATATGAAC-3' (sense)
5'-TTTACAGGTGATTCTACCTATGAATTTAGAAATTTC-3' (antisense)

TNF-α, Tm=55ºC

(T7)5'-GAACATCAACCTTCCCAAACGCCTC-3' (sense)
5'-ATTTCTCGCCACTGAATAGTGCCGCATTAC-3' (antisense)

IL-8, Tm=55ºC

(T7)5'-AATTCATTCTCTGTGGTATCCAAGAATC-3' (sense)
5'-CTAAAAACCCTGATTGAAATTTATCTAATAAAC-3' (antisense)

The PCR products were gel purified and used to transcribe biotinylated probes by using the T7 polymerase and biotin-labeled 14-CTP (Invitrogen). The recipe used to prepare the biotin 14-CTP nuclear mix was [10 mM ATP, 10 mM GTP, 10 mM UTP, 9 mM CTP, 1 mM biotin-14-CTP]. The in vitro transcription reaction mix used to make the biotinylated probes was:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 µl</td>
<td>1 µg PCR template (up to 11 µl with Rnase free water)</td>
</tr>
<tr>
<td>4 µl</td>
<td>5x transcription buffer</td>
</tr>
<tr>
<td>2 µl</td>
<td>100 mM DTT</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>RNasin</td>
</tr>
<tr>
<td>1 µl</td>
<td>Biotin-14-CTP nuclear mix (product of step 1 above)</td>
</tr>
<tr>
<td>1.5 µl</td>
<td>T7 RNA polymerase</td>
</tr>
</tbody>
</table>

20 µl Total volume

The in vitro reaction mix was incubated for 2 h at 37ºC, and was then treated with 2 µl 10X Dnase buffer and 1 µl Dnase (Rnase free). The reaction mix was incubated for 15 min at 37ºC, and then deactivated with 2 µl Dnase inactivation buffer (Ambion). Finally, 25 µl TE buffer (pH 8.0) was added to each reaction sample and the samples were
passed through a G-50 column as per the manufacturer's instructions (GE Healthcare, Hanover, PA). The RNA was precipitated and then dissolved 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 (15 µl) were incubated with 120 µg of cytoplasmic lysate for 30 min at room temperature using an end-over-end rotator. During the biotin-labeled synthetic RNA transcript and cytoplasmic incubation, 10 µl streptavidin-conjugated Dynabeads (Invitrogen) was pre-washed two times with 200 µl Solution A [0.1 M NaOH and 0.05 M NaCl] and once with 200 µl Solution B [0.1 M NaCl]. The beads were then resuspended in 10 µl 1X TENT buffer. The RNA-protein complexes were then harvested through incubation with the paramagnetic Dynabeads by adding the synthetic biotin-labeled RNA transcripts and lysate to the beads and letting them incubate for 30 min at room temperature in an end-over-end rotator. The RNA-protein complexes were then washed once in cold PBS and then resuspended in 45 µl 1X SDS and analyzed on a 10% SDS-PAGE gel. Western blotting using the HuR antibody described above was used to assess the binding of HuR protein to the synthetic RNA transcript (Figure 2.1). This protocol was previously described (Zou, Mazan-Mamczarz et al. 2006).

2.11 Endogenous RNA-protein binding assays

For IP of endogenous RNA-protein binding complexes, 2000 µg of cytoplasmic lysate that had been harvested in ice-cold PLB buffer [100 mM KCl, 5 mM MgCl2, 10 mM Hepes pH 7.0, 0.05% NP-40, 1 mM DTT, 100 U RNase OUT, 1X protease inhibitor cocktail] was pre-cleared in 15 µg IgG for 30 minutes on ice, followed by incubation for 30 min at 4°C with non-coated Protein A-Sepharose beads (Sigma-Aldrich, St. Louis, MO). The lysate was centrifuged for 1 min at 14,000 rpm and the supernatant was
Figure 2.1. The synthetic biotin-labeled RNA-protein binding assay.

PCR products were made for the 3'UTRs of interest. These PCR products contained the T7 promoter sequence on the 5' end. The T7 polymerase was used to transcribe biotin-labeled 3'UTR synthetic transcripts. These synthetic mRNAs were incubated with cytoplasmic lysate in order to allow proteins to bind to the 3'UTR sequence. Streptavidin Dynabeads were used to bind to the biotin of the labeled synthetic 3'UTRs, thus causing the 3'UTRs to pulldown any bound protein. The proteins were eluted and HuR binding to the synthetic 3'UTR was assessed via Western blotting.

Modified from a figure from Dr. Faoud Ishmael (Dept. of Biochemistry, Penn State College of Medicine).
saved in a clean 1.5 ml eppendorf tube. The 2000 µg of cytoplasmic lysate was then incubated for 2 h at room temperature with 50 % (v/v) suspension of Protein A-Sepharose beads that had been pre-coated with 30 µg of either mouse IgG (Invitrogen) or HuR antibodies overnight and resuspended in 700 µl NT2 buffer [50 mM Tris pH 7.4, 150 mM NaCl, 1 mM MgCl₂, and 0.05% NP-40], 10 µl 100 mM DTT, 10 µl RNase OUT, and 33 µl EDTA. The beads and lysate were then centrifuged at 14,000 rpm for 5 min at 4ºC. The pellet was washed 5 times with 1 ml aliquots of ice-cold NT2 buffer between spins (total of 5 spins and 5 washes). After the final wash the supernatant was removed and the beads were resuspended in 2.5 µl Proteinase K (Invitrogen), 1 µl 10% SDS, and 100 µl NT2 buffer. The beads were then incubated at 55 ºC for 30 min in a Thermomix set to 700 rpm. The beads were centrifuged at 14,000 rpm for 2 min at 4ºC. The supernatant was collected in a clean tube and the beads were washed again in 200 µl NT2 buffer and spun at 14,000 rpm for 2 min at 4ºC. Again the supernatant was removed and combined with the previously removed supernatant. The RNA was extracted using phenol-chloroform extraction in the presence of glycoblue and placed in -20ºC overnight. The following day the RNA was pelleted by centrifugation at 14,000 rpm for 30 min at 4ºC. After resuspending the RNA in 20 µl of water, the RNA was treated with 2 µl 10X Dnase reaction buffer and 1 µl Dnase for 30 minutes at 37ºC. The RNA was harvested again by phenol-chloroform extraction and resuspended in 20 µl Rnase-free water. The protocol used was previously described (Figure 2.2) (Lal, Mazan-Mamczarz et al. 2004). The RNA was measured through both conventional PCR and qRT-PCR. The ODC and Cyclophilin A primers used for qRT-PCR are described in Section 2.7. Primers for the full length ODC coding region for conventional PCR were:

ODC coding region (CR), Tm=54.0ºC

5’-CGAGAACCATTGAGCAGCCTTTAC-3’ (sense)

5’-CTACACATTGATCCTAGCAGAA-3’ (antisense)
Figure 2.2. Endogenous RNA-protein binding assay.

Protein A-Sepharose beads were pre-coated with HuR or IgG antibody. The beads were incubated with pre-cleared cytoplasmic lysate (see text for more details). The mRNAs bound to the HuR protein were immunoprecipitated using phenol-chloroform extraction methods. The level of specific mRNA product bound to either HuR or the non-specific IgG was assessed via conventional PCR and qRT-PCR.

Modified from a figure from Dr. Faoud Ishmael (Dept. of Biochemistry, Penn State College of Medicine).
The GAPDH and c-Myc products were also analyzed by conventional PCR as negative and positive controls respectively. The primers used for c-Myc were:

\[
\begin{align*}
\text{c-Myc 3'UTR}, & \quad \text{Tm}=50.2^\circ C \\
5'-' & \text{-ACTGACCTAACTCGAGGAGGAG-3}' (\text{sense}) \\
5'-' & \text{-GTATTTTTTCCAATTATTTAT-3}' (\text{antisense})
\end{align*}
\]

\[
\begin{align*}
\text{GAPDH coding region (CR), } & \quad \text{Tm}=62.2^\circ C \\
5'-' & \text{-CCAGCCTCGTCCCGTAGACAAAA-3}' (\text{sense}) \\
5'-' & \text{-TCGGCCTTGACTGTGCCTTGAA-3}' (\text{antisense}).
\end{align*}
\]

2.12 \textbf{M-fold}

To investigate the secondary structures of the fragment and deletion constructs used in the biotinylated-labeled RNA-protein binding assays, as well as investigate the secondary structures for the full length ODC 3'UTR, the M-fold website was used (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi). The RNA sequence was placed into the command box and the predicted secondary structures were generated. The secondary structure that was the most favorable, with the highest $\Delta G$, was chosen as the most probable secondary structure.

2.13 \textbf{Immunofluorescence Staining}

A5 and C5N cells were seeded onto glass coverslips that had been pre-coated with poly-L-lysine (Sigma-Aldrich) and placed in a 12 well plate at a density of 10,000 cells and 20,000 cells respectively. Cells grew to 70% confluency and were then fixed in 50/50 acetone/methanol solution at -20ºC for 5 min. The samples were incubated overnight at 4ºC in 5% goat serum in PBS. HuR antibody was placed on the samples (diluted 1:500 in PBS containing 0.1% BSA) for 2 h. The samples were then incubated
in Cy-2 secondary antibody at a 1:200 dilution in PBS containing 0.1% BSA for 1 h (Jackson Immunoresearch Laboratories, West Grove, PA). The coverslips were incubated with DAPI diluted to 1 µg/ml for 10 min at room temperature. The cells were washed and then mounted using Aqua Poly/Mount (Polysciences Inc., Warrington, PA). Slides were viewed through the Nikon Eclipse E800 microscope and pictures were taken using the Nikon ACT-1 Software (Nikon Instruments Inc.). Images were processed using Windows Photo Gallery (Microsoft, Redmond, WA).

2.14 RNA interference

A validated small interfering RNA (siRNA) duplex corresponding to the coding region of HuR and a negative control duplex were purchased from Dharmacon Research (Thermoscientific, Lafayette, CO). siHuR contained the sequence 5’ GGUUGAAUCUGCAAAGCUU 3’. For RNAi experiments, A5 cells were plated at a density of 17,000 cells per well in a 12 well plate. Forty-eight h later, the cells had reached 70% confluency. C5N cells were plated at a density of 90,000 cells per well in a 12 well plate and reached 70% confluency 16-24 h later. Upon 70% confluency, cells were transfected with 100 nM (final concentration) siRNA or a negative control siRNA using DharmaFECT1 reagent (Thermoscientific). The negative control and siRNA transfection were carried out according to the manufacturer’s protocol. Knockdown efficiency was assessed 72 h post transfection through qRT-PCR, Northern blot, or Western blot analysis. For Northern blot analysis and qRT-PCR, RNA was harvested using TRIzol extraction as described in Section 2.7. For Western blot analysis protein was harvested in 1X RIPA buffer and assessed as described in Section 2.2. ODC enzyme activity was tested in mock, negative control, or siHuR treated cells as described above in Section 2.4. ODC mRNA stability in mock, negative control, or siHuR treated cells was also assessed by treating each well with 10 µg/ml of
Actinomycin D. RNA was harvested at 0 h, 4 h, and 8 h, using TRIzol extraction. The ODC mRNA half-life was determined by both Northern blot analysis and qRT-PCR technology using the same methods and primers described in Sections 2.7 and 2.8.

Additionally, cell viability of A5 cells treated with the siHuR, the negative control, or mock treated was assessed via MTT assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolic compound that is reduced to a purple formazan product by reductase enzymes that reside in the mitochondria of living cells. The conversion of the tetrazolic compound to the purple formazan product is a read-out of mitochondrial activity and thus is directly correlated to cell viability. Seventy-two h post-siHuR treatment, A5 cells that had not been treated, mock treated cells, control siRNA treated cells, and siHuR treated cells were incubated with MTT as per the manufacturer’s instructions (Sigma). Briefly, MTT solution, dissolved in PBS, was added to each well in a 12 well plate (0.5 mg/ml final concentration) (Sigma). The plate was then incubated in a tissue culture incubator set to 37°C for 4 h. The solution was aspirated and the cells were solubilized in 2 ml DMSO. Upon homogeneity, 100 µl of each sample was assayed in triplicate in a 96-well plate. The absorbance was read at a 570 nm wavelength using a SPECTRAmax PLUS384 plate reader (Molecular Devices Corporation, Sunnyvale, CA). Cell viability was calculated as a percent of the optical density (OD) value of untreated cells.

2.15 Rapamycin experiments

To test the effects of Rapamycin on ODC enzyme activity and ODC mRNA steady-state levels, A5 cells were seeded at a density of 22,500 cells per 6 cm plate. Upon 70% confluency, the cells were treated with 100 nM Rapamycin (dissolved in DMSO) (Tocris Bioscience, St. Louis, MO) in serum free media. Control cells were treated with DMSO only. At various time points after the addition of Rapamycin (2, 6, 8,
10, 12, and 24 h), RNA was extracted as described in Section 2.7 and qRT-PCR was used to assess the quantity of RNA present as described in Section 2.8. Additionally, samples were taken for ODC enzyme activity assays at time points 3, 6, 8, 10, and 24 h. The ODC enzyme activity was measured as described in Section 2.4.

To investigate the effect of Rapamycin on ODC mRNA stability, A5 cells were plated at a density of 150,000 cells per well in a 6 well plate. Upon 70% confluency (16-24 h later), 100 nM Rapamycin (dissolved in DMSO) was added to each well and DMSO was used to treat the control cells. Twenty-four h after treatment, Actinomycin D was added to each well (10µg) and RNA was extracted at 0 h, 4 h, and 8 h post-Actinomycin D treatment. RNA was extracted via the TRIzol method as described in Section 2.7. The mRNA half-life was determined by exponential curve fit analysis.

To investigate the effect of Rapamycin on HuR localization, A5 cells were seeded at a density of 10,000 cells onto poly-L-lysine coated coverslips in a 6 well plate. Upon 70% confluency, cells were treated with 100 nM (diluted in DMSO) Rapamycin, and control cells were treated with DMSO. At various time points (0, 1, 4, 8, and 24 h) the cells were fixed onto the coverslips and HuR localization was determined as described in Section 2.13.

2.16 TPA experiments

C5N cells were plated at a density of 35,000 cells onto poly-L-lysine coated coverslips that had been placed in 12 well plate. Upon 70% confluency, cells were treated with 15.4 nM TPA (diluted in DMSO) while control cells were treated with DMSO only. Cells were fixed at the time points 1 h, 4 h, 6 h, and 24 h after TPA or DMSO treatment, and HuR localization was determined using the same technique described in Section 2.13.
2.17 AICAR experiments

A5 and C5N cells were seeded onto poly-L-lysine glass cover slips placed in 3.5 cm plates at a density of 10,000 and 15,000 cells respectively. Upon 70% confluency cells were serum starved for 24 h. 2 mM and 4 mM AICAR (dissolved in DMSO) (Cell signaling) was added to each experimental plate, while control cells were treated with DMSO. Four h after AICAR treatment, cells were fixed and HuR localization was determined using the same protocol described in Section 2.13.

2.18 ODC 3’UTR and 5’ UTR luciferase assays

The ODC 3’UTR was cloned into the pGL3-Control vector (Promega) and placed downstream of the Firefly luciferase reporter gene while the ODC 5’UTR was cloned into the pGL3-Control vector (Promega) and placed upstream of the Firefly luciferase reporter gene. These plasmids are denoted pODC3’UTRLuc and pODC5’UTRLuc respectively. A 338 base pair truncation of the proximal ODC 3’UTR denoted herein as ARE03 and a 178 base pair truncation of the proximal ODC 3’UTR denoted ARE02, were cloned into the pGL3 Control vector (Promega) downstream of the Firefly luciferase reporter gene.

To investigate the influence the ODC 3’UTR and ODC 5’UTR had on luciferase activity, A5 and C5N cells were seeded at 20,000 and 35,000 cells per 6 cm plate respectively. Upon 70% confluency, the cells were transfected with either 2 µg per plate of pODC3’UTRLuc, pODC5’UTRLuc, or pGL3 Control using the Lipofectamine 2000 transfection reagent as per the manufacturer’s protocol (Invitrogen). Mock transfected cells were treated with Lipofectamine 2000 only. The pRL-SV40 Renilla reporter plasmid was transfected at 0.2 µg per plate in order to act as a transfection efficiency control. Forty-eight h post-transfection, cells were harvested in 1X Passive Lysis Buffer (Promega) and assayed using the Dual-Luciferase Kit as per manufacturer’s instructions.
For each sample, the Firefly luciferase activity was normalized to the Renilla luciferase activity, and the data were expressed as the Firefly/Renilla ratio. The data were normalized to the pGL3 Control.

To investigate the influence the proximal end of the ODC 3’UTR had on luciferase activity, A5 and C5N cells were seeded onto 6 cm plates at a density of 150,000 cells per plate. Upon 70% confluency, the cells were transfected with 2 µg per plate of either pODC3’UTRLuc, ARE02, ARE03, or pGL3 Control. Cells were transfected using Lipofectamine 2000 as per the manufacturer’s protocol (Invitrogen). The pRL-SV40 Renilla reporter was transfected at 0.2 µg per plate in order to act as a transfection efficiency control. Forty-eight h post-transfection cells were assayed using the Dual-Luciferase Kit as described above. For each sample, the Firefly luciferase activity was normalized to the Renilla luciferase activity, and the data were expressed as the Firefly/Renilla ratio. The data were normalized to the pGL3 Control.

2.19 Site-directed mutagenesis of the AUUUA site in the ODC 3’UTR

Site-directed mutagenesis was conducted on the AUUUA site in the ODC 3’UTR using the Stratagene Quikchange Site-directed Mutagenesis Kit as per the manufacturer’s instructions (Stratagene, La Jolla, CA). The primers used to create the AUUUA to GGGUA mutation were:

HuR site-directed primer

5’-GGCATTTGGGGGACCGGTAACTTAATTACTGCTAGTTTGG-3’ (sense)
5’-CCAAAACTAGCAGTAATTAAGTTACCGGTCCCCCCAAATGCC-3’ (antisense)

The ARE02 truncation plasmid, described in section 2.18, was used as the template for site-directed mutagenesis. The AUUUA to GGGUA mutation was validated by sequencing. To conduct biotinylated pulldowns (described in Section 2.10) using the
mutated ARE02 or wild-type ARE02, the following primers were used:

ARE02 primers, Tm=63°C

(T7)5'-ATGCCATTCTTGTAGCTTTGC-3' (sense)

5'-CAAAATTTTCTAGGAAACACAGTAAC-3' (antisense).

To investigate the influence the AUUUA sequence had on luciferase activity, C5N cells were seeded onto 6 cm plates at a density of 150,000 cells per plate. Upon 70% confluency, the cells were transfected with 2 µg per plate of either pODC3'UTRLuc, ARE02 wild-type, ARE02 mutant, or pGL3 Control. Cells were transfected using Lipofectamine 2000 as per the manufacturer’s protocol (Invitrogen). The pRL-SV40 Renilla reporter was transfected at 0.2 µg per plate in order to act as a transfection efficiency control. Forty-eight h post-transfection cells were assayed using the Dual-Luciferase Kit as described above. For each sample, the Firefly luciferase activity was normalized to the Renilla luciferase activity, and the data were expressed as the Firefly/Renilla ratio. The data were normalized to the pGL3 Control.

2.20 Statistics

Results are expressed as means ± standard errors (SE) from three to nine samples. Autoradiographic and immunoblotting were repeated up to three times. Statistical analysis was performed using Student’s t test. P values of <0.05 were considered significant.
CHAPTER 3
Characterization of a mouse non-melanoma skin cancer model used to study ornithine decarboxylase (ODC) regulation

3.1 Introduction

Ornithine decarboxylase (ODC) is one of the rate-limiting enzymes in the polyamine biosynthetic pathway [Reviewed in (Wallace, Fraser et al. 2003; Pegg 2006)]. Polyamines, small, ubiquitous polycations, are necessary for normal cell growth and development. Thus, the ablation of ODC is embryonic lethal in mice (Fozard, Part et al. 1980; Pendeville, Carpino et al. 2001). Due to their charge, polyamines are able to bind to RNA, DNA, and proteins and thereby influence gene expression [Reviewed in (Igarashi, Sakamoto et al. 1982; Wallace 2000; Wallace, Fraser et al. 2003)]. Both polyamine content and ODC enzyme activity are tightly regulated in cells, and ODC is induced in response to a variety of proliferative stimuli by alterations in its transcription, translation, and protein degradation (Bello-Fernandez, Packham et al. 1993; Wallon, Persson et al. 1995; Shantz and Pegg 1999; Zhao, Kumar et al. 2000; Zhao and Butler 2001; Shantz 2004; Mangold 2006; Pegg 2006).

A link between neoplastic transformation and increased ODC enzyme activity, as well as increases in intracellular putrescine and spermidine, has been well documented in animal models of skin carcinogenesis and other epithelial tumors (O'Brien 1976; Pegg 1988; Auvinen, Paasinen et al. 1992; O'Brien, Megosh et al. 1997; Peralta Soler, Gilliard et al. 1998; Smith, Trempus et al. 1998; Gerner and Meyskens 2004; Feith, Bol et al. 2005). The increase in ODC activity during tumor promotion has been attributed to both increased transcription of the Odc gene and translation of the ODC mRNA [Reviewed in (Shantz 2004; Pegg 2006). Odc transcription is stimulated by growth factors and tumor promoting agents (Katz and Kahana 1987; Verma, Hsieh et al. 1988). In fact, the Odc
promoter region contains many known transcription factor binding sites, including binding sites for the oncogene c-Myc (Auvinen, Jarvinen et al. 2003). Translation of ODC has been shown to be regulated by the Ras pathway, a pathway that is often upregulated during neoplastic transformation (Origanti and Shantz 2007).

To date, most work describes the correlation between ODC induction and skin tumorigenesis in vivo (O'Brien, Simsiman et al. 1975; O'Brien 1976; Feith, Shantz et al. 2001). For example, Keratin 6/ODC transgenic mice display increased ODC enzyme activity and high putrescine levels in both the dermis and epidermis. Furthermore, these mice do not require TPA treatment to promote tumor formation after DMBA initiation, demonstrating that ODC overexpression is sufficient for skin tumor promotion (O'Brien, Megosh et al. 1997). Clifford et al. showed that ODC overexpressing mouse BK-1 primary keratinocyte cells did not yield tumors when injected subcutaneously into nude mice. Similarly, cells that contained activated Ha-ras were unable to form tumors in nude mice when injected subcutaneously. However, when ODC was overexpressed in skin epidermal cells with activated Ha-ras, tumors formed, showing that ODC overexpression contributes to tumor development in epithelial cells (Clifford, Morgan et al. 1995). Moreover, overexpression of the endogenous inhibitor of ODC, AZ1, inhibits tumor formation in mice that are treated with DMBA and TPA (Feith, Shantz et al. 2001). These studies suggest that ODC is both necessary and sufficient for skin tumor development. The studies described here are the first comprehensive in vitro studies looking at ODC enzyme activity and regulation during skin tumor development.

The in vitro mouse keratinocyte data from our studies not only support the previous in vivo results, but also enhance our understanding of ODC regulation during skin tumor development. Our model is comprised of two cell lines which represent the progression of non-melanoma skin cancer. A non-transformed cell line that contains wild-type Ha-ras as well as wild-type p53 is denoted as C5N. The transformed spindle
carcinoma cell line is denoted as A5. These cells were isolated from the tumors of mice that had been subjected to the multi-stage chemical carcinogenesis protocol. In addition to DMBA treatment, these mice were treated with the tumor promoting agent, TPA, weekly for 40 weeks. A5 cells contain 1 wild-type allele and 2 mutant alleles for Ha-ras at codon 61 as well as a mutated p53 gene (Zoumpourlis, Solakidi et al. 2003).

The studies described here in Chapter 3 examine the morphological characteristics of C5N and A5 cells. We show that ODC mRNA half-life as well as ODC enzyme activity differ between these two cell lines. The A5 cells show an induction of ODC enzyme activity as well as a dramatic increase in ODC mRNA stability when compared to the C5N keratinocytes. Furthermore, the level of putrescine, the product of the ODC reaction, is higher in A5 cells when compared to the C5N normal keratinocytes, suggesting that induction of ODC is key for the development of non-melanoma skin cancer, in agreement with the transgenic models described above.

3.2 Results

3.2.1 The effect of neoplastic transformation on the morphology and growth rate of keratinocyte-derived cell lines.

Based on previous studies in which ODC enzyme activity was assessed during neoplasia, we hypothesize that ODC enzyme activity will be elevated in A5 spindle carcinoma cells when compared to C5N keratinocytes, and that the increase in ODC enzyme activity is due to an upregulation of transcription, increased stability of the ODC mRNA, more efficient translation, and/or decreased degradation of ODC in A5 cells. In order to determine the effects of neoplastic transformation on both ODC and polyamine regulation in the skin, C5N non-transformed keratinocytes and A5 spindle carcinoma cells were utilized. C5N non-transformed keratinocytes displayed typical epithelial morphology, being flat and cuboidal. A5 cells, harvested from spindle cell carcinoma
tumors, exhibited a fibroblastic morphology, typical of cells that have undergone an epithelial to mesenchymal transition (Figure 3.1). The A5 cells were shown previously to be aggressively tumorigenic, causing the development of tumors 4 weeks after injection into nude mice (Zoumpourlis, Solakidi et al. 2003). Moreover, the doubling time for C5N cells was longer when compared to the doubling time in A5 spindle carcinoma cells. C5N cells displayed a doubling time of approximately 25 hours whereas A5 cells displayed a doubling time of approximately 19 hours (Table 3.1). As non-transformed cells usually grow more slowly than transformed cells, our data further suggest a change in phenotype.

3.2.2 ODC enzyme activity, ODC mRNA stability, and putrescine levels are increased in transformed cells when compared to non-transformed keratinocyte-derived cells.

To assess the effect of neoplastic transformation on ODC, we conducted an ODC enzyme activity assay. We observed a 4-fold change in ODC enzyme activity between the two cell lines (Figure 3.2), with C5N keratinocytes displaying low ODC enzyme activity while the A5 cells exhibited a significant induction in ODC enzyme activity. Polyamine levels were assessed to determine the effect ODC induction had on intracellular polyamine content because a correlation between high ODC enzyme activity and increased levels of putrescine has been recorded in skin carcinogenesis studies (O'Brien 1976; Rosen, Gajic et al. 1990; O'Brien, Megosh et al. 1997; Tabib and Bachrach 1998; Feith, Bol et al. 2005; Feith, Origanti et al. 2006). Our results reveal a significant increase in intracellular putrescine in A5 cells when compared to C5N cells. Additionally, a trend towards higher spermidine levels in A5 cells was exhibited (P=0.2206). No change in spermine levels was recorded between the two cell lines (Table 3.2). These data are in agreement with other models that showed a similar level of ODC induction (O'Brien, Megosh et al. 1997; Peralta Soler, Gilliard et al. 1998; Feith, Origanti et al. 2006).
Figure 3.1. Morphology of non-transformed C5N keratinocytes and A5 spindle carcinoma cells.

C5N cells, non-transformed keratinocytes, display a classical epithelial phenotype being cuboidal, with a cobblestone appearance. A5 cells, isolated from spindle cell carcinoma tumors of mice that had been treated with DMBA and TPA display a fibroblastic phenotype. Pictures were taken under light microscopy at a 20X magnification.
Table 3.1. C5N cells exhibit a longer generation time than A5 spindle carcinoma cells.

C5N and A5 cells were seeded at a density of 2000 cells per cm$^2$ on a 24 well plate. The number of cells per cm$^2$ was measured at time 0 h. The cell density was then measured daily until stationary phase was reached. Cells were counted using a Coulter Counter. The log of cells per cm$^2$ was plotted against time and the generation times were determined using exponential curve fit analysis. This experiment was done in duplicate with reproducible results.
Figure 3.2. ODC enzyme activity is increased in transformed mouse spindle carcinoma cells (A5) when compared to non-transformed keratinocytes (C5N).

ODC enzyme activity was determined in C5N cells and A5 cells using a radiometric technique as previously described in the Materials and Methods chapter. Briefly, cells were harvested during log-phase growth in ODC enzyme buffer (72 h). Values are the means ± S.E. from 10 plates. **P<0.005 when comparing the two cell lines.
Table 3.2. Non-transformed C5N keratinocytes display lower levels of putrescine and spermidine when compared to A5 spindle carcinoma cells.

Putrescine, spermidine, and spermine levels were measured as described in the Materials and Methods chapter. Values are the means ± S.E. *P<0.05. (n=6-19).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Putrescine (nmoles/mg protein)</th>
<th>Spermidine (nmoles/mg protein)</th>
<th>Spermine (nmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5N</td>
<td>1.13 ± 0.35</td>
<td>7.56 ± 1.69</td>
<td>5.60 ± 1.35</td>
</tr>
<tr>
<td>A5</td>
<td>3.58 ± 0.97 *</td>
<td>10.38 ± 1.39</td>
<td>5.43 ± 1.18</td>
</tr>
</tbody>
</table>

*Table 3.2. Non-transformed C5N keratinocytes display lower levels of putrescine and spermidine when compared to A5 spindle carcinoma cells.*

Putrescine, spermidine, and spermine levels were measured as described in the Materials and Methods chapter. Values are the means ± S.E. *P<0.05. (n=6-19).*
To further explore the mechanism of increased ODC enzyme activity, several assays were conducted to measure Odc transcription and mRNA stability. Northern blot analysis and qRT-PCR revealed a trend towards higher steady-state ODC mRNA in A5 cells when compared to C5N cells; however, this trend was not statistically significant (P>0.2) (Figure 3.3). Furthermore, promoter studies using the pODCLuc vector, a vector in which the mouse Odc promoter is upstream of a Firefly luciferase reporter gene, showed that transcription was statistically higher in C5N normal keratinocytes when compared to A5 spindle carcinoma cells (Figure 3.4). These results suggest that the ODC mRNA half-life is shorter in C5N cells when compared to A5 cells.

We decided to investigate the stability of the ODC mRNA in order to better understand the mechanism behind the increased ODC enzyme activity in the A5 cells. Using both Northern blot analysis with subsequent band quantitation and qRT-PCR, we determined that the ODC mRNA half-life was 4 h in the C5N keratinocytes compared to 15 h in A5 cells (Figure 3.5). These data show that ODC mRNA is markedly less stable in the normal keratinocytes than in carcinoma cells, and that the observed change in ODC stability alone could account for the difference in the ODC enzyme activity between the two cell lines. Thus, we decided to further investigate the mechanism behind the enhanced ODC mRNA stability in A5 cells, and we report these findings in Chapter 4.

3.3 Discussion

The data described here agree with previous in vivo and in vitro findings that show ODC enzyme activity is induced during carcinogenesis (O'Brien 1976; Auvinen, Paasinen et al. 1992; Peralta Soler, Gilliard et al. 1998). In a study using NIH-3T3
Figure 3.3. ODC steady-state mRNA is not statistically different between C5N and A5 cells.

ODC mRNA steady-state levels were determined by both Northern blot analysis and qRT-PCR as described in the Materials and Methods chapter. A, Steady-state ODC mRNA levels determined by Northern blot analysis. ODC mRNA levels were normalized to a Cyclophilin A control (n=2). B, Steady-state ODC mRNA levels determined by qRT-PCR. ODC mRNA levels were normalized to Cyclophilin A (n=3-5). C, Steady-state ODC mRNA levels determined by combining qRT-PCR and Northern blot analysis data. ODC mRNA values were normalized to Cyclophilin A. Values are means ± S.E. from 4 separate experiments. (P>0.2).
Figure 3.4. ODC transcription is significantly higher in non-transformed C5N cells when compared to transformed A5 cells.

The level of ODC transcription was assessed by using pODCLuc in which the mouse ODC promoter was placed upstream of a Firefly luciferase reporter gene. Luciferase assays were conducted as described in the Materials and Methods chapter. pGL3 is a control plasmid in which the Firefly luciferase reporter gene was under the control of a SV40 promoter. The data were normalized to a Renilla-driven luciferase plasmid. The pGL3 data were set to 1 and the pODCLuc data are shown as a percent of the pGL3 control data. Values are means ± S.E. from 9 plates. *P<0.05 when comparing the ODC promoter activity between the C5N and A5 cell lines.
Figure 3.5. ODC mRNA stability is significantly higher in A5 spindle carcinoma cells (11-15 h) when compared to C5N normal keratinocytes (4-5 h).

ODC mRNA half-life was determined using 10 µg Actinomycin D/ml. Total RNA was isolated at the indicated times and the levels of ODC mRNA and Cyclophilin A mRNA were assessed via Northern blot analysis or qRT-PCR according to the protocols outlined in the Materials and Methods Chapter. ODC mRNA levels were normalized to a Cyclophilin A control for each time point. A, Example of Northern blot for ODC mRNA. B, Curve fit analysis for determining the ODC mRNA half-life for the Northern blot in panel A. C, Curve fit analysis for determining the ODC mRNA half-life using one qRT-PCR experiment. The samples for the qRT-PCR graph are different from the samples used in Panels A and B. D, ODC mRNA half-life using data from both qRT-PCR (n=2) and Northern blot experiments (n=2). Values are the means ± S.E. from 4 separate experiments.
fibroblasts, Moshier et al. overexpressed ODC and found an increase in both ODC activity and ODC mRNA levels. The increased ODC mRNA and protein levels correlated with a transformed phenotype as cells overexpressing ODC displayed increased growth rates, anchorage independent growth, and induced tumors in nude mice (Moshier, Dosescu et al. 1993). These and previously described in vivo studies emphasize the importance of ODC regulation and demonstrate that the upregulation of ODC is an important step during neoplastic transformation.

Our data expands upon previous findings by using mouse epithelial cell lines that exhibit a non-transformed phenotype (C5N) or a transformed phenotype (A5). The mouse epithelial cell model used in these studies is more physiologically relevant to study tumorigenesis because most tumors are epithelial in origin. Many of the previous studies investigating ODC induction and cancer used NIH-3T3 fibroblasts, a cell line with non-epithelial behavior and morphology (Auvinen, Paasinen et al. 1992; Moshier, Dosescu et al. 1993; Clifford, Morgan et al. 1995; Shantz and Pegg 1998). Thus, the model described here is more conducive for drawing conclusions about epithelial cancers. The model used in these studies to investigate ODC regulation during neoplastic transformation is novel because previous studies looking at ODC regulation in vitro often use exogenous ODC (Moshier, Dosescu et al. 1993; Clifford, Morgan et al. 1995). Conversely, the C5N and A5 cell lines do not contain foreign DNA and use the multi-stage chemical carcinogenesis model to induce ODC enzyme activity. One caveat to this model is that in addition to ODC upregulation, other genes are likely differentially mutated or deregulated in the two cells lines, making it more challenging to discern the specific effects of ODC induction in these cells. However, this model is more physiologically relevant than one in which one gene is transfected into a cell line because it more closely mimics the actual process of neoplastic transformation. Furthermore, keratinocyte-derived cell lines are an ideal model for studying non-
melanoma skin cancer because keratinocytes are the progeny of stem cells located within the bulge region of the hair follicle which are the targets of chemical carcinogens such as DMBA [Reviewed in (DiGiovanni 1992)]. While other cell lines may prove useful for studying the process of epithelial neoplastic transformation, the C5N and A5 cell lines are specific for studying non-melanoma skin cancer.

The studies described here characterize ODC regulation in two mouse keratinocyte-derived cell models during the development of skin carcinomas. C5N non-transformed keratinocytes displayed low ODC enzyme activity when compared to transformed A5 spindle carcinoma cells (Figure 3.2). C5N cells also exhibited lower levels of putrescine and spermidine when compared to A5 cells (Table 3.2). As ODC is regulated at the level of transcription, we sought to investigate the rate of Odc transcription in these cells by using the pODCLuc vector [Reviewed in (Pegg 2006)]. Interestingly, these results showed that Odc transcription was slightly higher in non-transformed C5N cells when compared to transformed A5 cells (Figure 3.4). Moreover, when we investigated the steady-state mRNA levels in C5N and A5 cells, we saw no statistically significant change in ODC mRNA between the two cell lines, although A5 cells did display a trend towards higher ODC steady-state mRNA levels (Figure 3.3). The higher ODC enzyme activity in A5 cells can be explained several ways. ODC is not known to be activated via post-translational modifications (Rosenberg-Hasson, Strumpf et al. 1991). C5N cells, although exhibiting higher Odc transcription, could have less stable ODC mRNA when compared to A5 cells. This would account for the higher Odc transcription in C5N cells and the similar steady-state mRNA levels between the two cell lines. Secondly, the ODC mRNA can be translated less efficiently in C5N cells when compared to A5 cells. This would explain the similar steady-state mRNA levels between the two cell lines with the C5N displaying lower ODC enzyme activity, as ODC enzyme activity correlates with ODC protein levels in all models tested thus far. Thirdly, ODC
protein could be degraded at a faster rate in C5N cells when compared to A5 cells thus causing the decrease in ODC enzyme activity in C5N cells. Although it would be simple to assume that only one of the proposed mechanisms is the cause for the change in ODC enzyme activity between the two cell lines, it is likely that a combination of the described regulatory events is causing the induction of ODC enzyme activity in the transformed A5 cells.

We sought first to investigate the ODC mRNA stability in the C5N and A5 cells. The ODC mRNA half-life was approximately 4-fold shorter in non-transformed C5N cells when compared to A5 cells (Figure 3.5). This change in ODC mRNA stability can explain why C5N cells have a higher rate of Odc transcription while having a similar level of steady-state ODC mRNA when compared to the transformed A5 cell line. Moreover, the decreased ODC mRNA stability in non-transformed C5N cells can explain the lower ODC enzyme activity in C5N cells. Only one other study has been published that addresses changes in ODC mRNA stability as a means of regulating ODC enzyme activity. ODC mRNA stability changes have previously been found to influence the ODC enzyme activity of Erhlich ascites tumor cells. Stimulation of these cells with serum caused an increase in ODC enzyme activity that correlated with an enhanced ODC mRNA stability. Cells that exhibited low ODC enzyme activity displayed lower ODC mRNA stability (Wallon, Persson et al. 1995). These studies support the notion that ODC is regulated, in part, at the level of mRNA stability and that this can influence ODC enzyme activity.

The data in this study support the hypothesis that ODC enzyme activity is induced during skin neoplastic transformation. Enhanced Odc transcription in the transformed cells did not occur; interestingly, the C5N cells showed stronger Odc promoter activity. Instead our results show that the induction of ODC in A5 spindle carcinoma cells is in part, due to an increase in the ODC mRNA stability. The
mechanism of ODC mRNA stability enhancement in transformed cells will be the focus of Chapter 4.
CHAPTER 4

The cytoplasmic accumulation of the RNA binding protein HuR stabilizes the ornithine decarboxylase mRNA transcript in a mouse non-melanoma skin cancer model

4.1 Introduction

As described in Chapter 3, ODC enzyme activity is upregulated in spindle carcinoma cells when compared to non-transformed keratinocytes. These data affirm previous findings in which ODC enzyme activity was increased in tumors when compared to normal tissue (O'Brien 1976; Feith, Bol et al. 2005). In order to determine the mechanism of ODC induction in A5 transformed cells, we first investigated mRNA stability. We showed that in addition to elevated ODC enzyme activity, A5 cells displayed increased ODC mRNA stability when compared to C5N keratinocytes. To further explore a mechanism by which ODC mRNA stability was enhanced in A5 cells we decided to look at RNA binding proteins (RBPs) because changes in the intracellular polyamine pool have been shown to affect the localization of the RBP HuR (Zou, Liu et al. 2008). The altered localization of HuR can influence the stability of its target mRNA transcripts (Zou, Mazan-Mamczarz et al. 2006; Xiao, Rao et al. 2007).

Control of mRNA decay is a rapid means of regulating the expression of mRNAs [Reviewed in (Dreyfuss, Kim et al. 2002)]. RBPs bind to adenosine- and uracil-rich elements (AREs), sequences classically located within the 3’UTR of labile mRNAs [Reviewed in (Parker and Sheth 2007)]. These sequences behave as cis-acting elements, and are located in numerous proto-oncogene, cytokine, and transcription factor mRNAs [Reviewed in (Audic and Hartley 2004)]. Three categories of RBPs have been defined: those that stabilize mRNA, those that destabilize mRNA, and those that can both stabilize and destabilize mRNA [Reviewed in (Guhaniyogi 2001)]. Additionally,
it has been shown that some RBPs can alter the translational efficiency of their bound mRNA transcript (Mazan-Mamczarz, Galban et al. 2003; Liu, Rao et al. 2009).

The RBP HuR (human antigen R) belongs to the family of Hu proteins that are highly homologous to the ELAV family of proteins in *Drosophila melanogaster*. Unlike its family members HuB, HuC, and HuD, which are expressed exclusively in neurons, HuR is ubiquitously expressed (Ma, Cheng et al. 1996). The HuR protein contains three RNA recognition motifs (RRMs) (Ma, Cheng et al. 1996; Brennan and Steitz 2001). RRM 1 and RRM2 have been shown to recognize AREs located within the RNA, while it has been suggested that RRM3 recognizes the poly(A) tail of the mRNA transcript (Ma, Cheng et al. 1996; Ma, Chung et al. 1997; Brennan and Steitz 2001). The hinge region of HuR, located between RRM2 and RRM3, contains a nucleocytoplasmic shuttling sequence. Both a nuclear localization signal and nuclear export signal are contained in this region (Fan and Steitz 1998). HuR is mainly nuclear, but has been shown to shuttle between the nucleus and cytoplasm in response to various stress stimuli such as UVB irradiation and H$_2$O$_2$ (Kuwano, Kim et al. 2008; Zhang and Bowden 2008). To date, only a few upstream signaling cascades have been shown to post-translationally modify HuR and cause it to shuttle into the cytoplasm [Reviewed in (Doller, Pfeilschifter et al. 2008)].

In the cytoplasm, HuR can stabilize and/or influence the translational efficiency of its bound mRNA transcript (Meng, King et al. 2005; Kuwano, Kim et al. 2008; Mazan-Mamczarz, Hagner et al. 2008; Tong and Pelling 2009). HuR has been shown to bind to and stabilize numerous transcripts whose overexpression contribute to tumorigenesis, such as TSP-1, COX-2, and ProTα (Lal, Kawai et al. 2005; Mazan-Mamczarz, Hagner et al. 2008; Zhang and Bowden 2008). Given that HuR is a stabilizing RBP and that its cellular localization is influenced by the intracellular polyamine content, we decided to investigate whether HuR binds to the ODC mRNA transcript in our keratinocyte-derived cell lines (Zou, Mazan-Mamczarz et al. 2006; Xiao, Rao et al. 2007; Zou, Liu et al. 2008).
The studies described here will test the hypothesis that the RBP HuR binds to and increases the stability of the ODC mRNA in A5 spindle carcinoma cells.

The mouse keratinocyte cell model used in these studies is comprised of two cell lines: C5N and A5 cells, which have been previously described in Chapter 3 (Zoumpourlis, Solakidi et al. 2003). Here we investigate the mechanism for the marked increase in ODC mRNA stability in A5 cells when compared to C5N cells. Using both in vitro and intracellular assays, we show that HuR associates with the 3′UTR of ODC in transformed cells, but not in normal keratinocytes, and causes the ODC transcript to be stabilized. We further demonstrate that the binding status of HuR to the ODC mRNA transcript correlates with HuR localization, as HuR is exclusively nuclear in C5N cells but is both nuclear and cytoplasmic in A5 cells. Thus, these studies show for the first time that ODC is post-transcriptionally regulated by the stabilizing RBP HuR in a mouse non-melanoma skin cancer model. We also demonstrate that HuR not only binds to ODC mRNA in A5 cells but also binds to the 3′UTRs of other mRNAs associated with neoplastic transformation such as COX-2, TNF-α, and VEGF.

4.2 Results

4.2.1 HuR is able to bind to the ODC 3′UTR in A5 spindle carcinoma cells, but not C5N keratinocytes.

HuR has been shown to preferentially bind to ARE sequences that are located in the 3′UTR of the target mRNA transcript (Ma, Cheng et al. 1996; Hinman and Lou 2008). Upon examination of the mouse 3′UTR for ODC, one classical AUUUA ARE was identified (Figure 4.1). A biotinylated pulldown assay was conducted to determine whether HuR could bind to synthetic transcripts corresponding to the full-length ODC 3′UTR or the ODC coding region (Refer to Figure 2.1). The data show that cytoplasmic extracts of C5N cells demonstrate no measurable binding of HuR to either the ODC
**Figure 4.1.** The ODC 3’UTR contains a classical adenosine- and uracil-rich element (ARE).

The full length ODC 3’UTR sequence is shown. A classical AUUUA ARE is highlighted in gray.
3'UTR or coding region. Conversely, cytoplasmic A5 extracts exhibit strong HuR binding to the ODC 3'UTR biotinylated probe (Figure 4.2A, 4.2B). The difference in binding between C5N and A5 cells was not due to a lack of HuR in the C5N cells, because whole-cell lysates revealed that the relative level of HuR protein was similar between the two cell lines (Figure 4.2C). Together, these data show that HuR is present in both cell lines, but that only HuR present in the A5 cytoplasm is able to bind to the 3'UTR of ODC.

The endogenous intracellular association of ODC mRNA and HuR was assessed through immunoprecipitation of HuR in an assay that allows the association between HuR and its target mRNAs in cytoplasmic ribonucleoprotein (RNP) complexes to be maintained (Refer to Figure 2.2). In agreement with the biotinylated pulldown data, little ODC mRNA was immunoprecipitated from the C5N keratinocyte cell line cytoplasmic lysate, but a strong ODC band was detected in the immunoprecipitated material from A5 cells, as measured by both conventional PCR and qRT-PCR (Figures 4.3A and 4.3B). In fact, the qRT-PCR revealed a 9-fold increase in ODC mRNA in the A5 pulldown material when compared to the C5N. GAPDH was present at very low levels in both lysates. This is in agreement with previously reported results, as GAPDH mRNA can act as a partial contaminant in IP materials (Abdelmohsen, Pullmann et al. 2007). The positive control c-Myc mRNA associated strongly with HuR protein in A5 lysates (Figure 4.3A) (Liu, Rao et al. 2009).

4.2.2 HuR binds to a sequence on the ODC 3'UTR of A5 spindle carcinoma cells between bases 1851 and 2151.

HuR binding to the ODC 3'UTR is a novel finding because ODC mRNA has never been shown to bind to an RBP. To determine a more specific location of the HuR binding site in the 3'UTR of ODC, we created overlapping biotinylated probes of approximately 150-200 bases in length that scanned the entire ODC 3'UTR (Figure
Figure 4.2. The RBP HuR binds to the ODC 3'UTR in transformed A5 spindle carcinoma cells but is unable to bind to the ODC 3'UTR in non-transformed C5N keratinocytes.

A, Confirmation of a pure cytoplasmic extract. Both C5N and A5 cells were fractionated as described in the Materials and Methods chapter. To confirm purity, the cytoplasmic extracts were tested for nuclear contamination (Histone H3) and a cytoplasmic marker (α/β Tubulin). B, A5 cytoplasmic fractions demonstrate HuR binding to the ODC 3'UTR whereas no binding is exhibited in C5N cytoplasmic extracts. C5N and A5 cytoplasmic fractions were used in a biotin pulldown reaction in which 15 µl of biotin labeled ODC 3'UTR (3'UTR) or coding region (CR) was incubated with 120 µg of the cytoplasmic fraction. The pulldown material was assessed for HuR binding by Western blot analysis. A representative blot is shown. This experiment was done in duplicate with reproducible results. C, C5N and A5 whole-cell lysates display equal amounts of the HuR protein. 1X RIPA buffer was used to extract total cellular protein. HuR levels in C5N and A5 whole-cell lysates were measured by Western blot analysis. Equal loading was assessed by GAPDH. This experiment was done in triplicate with reproducible results.
Figure 4.3. The A5 cytoplasmic fraction displays endogenous HuR binding to the ODC mRNA whereas the C5N cytoplasmic fraction does not display HuR binding to the ODC mRNA.

A. The A5 cytoplasmic fraction exhibits HuR binding to the ODC mRNA transcript as shown by conventional PCR. The methods used for the mRNP assay are described in the Materials and Methods chapter. A5 cells exhibit HuR binding to the ODC mRNA and c-Myc positive control. GAPDH PCR was used as a negative control. This experiment was done in triplicate with reproducible results. B. The A5 cells demonstrate binding of HuR to the ODC mRNA. qRT-PCR was used as described in the Materials and Methods chapter, and showed that HuR binds to the ODC message in A5 spindle carcinoma cells. The ODC data were normalized to Cyclophilin A and the C5N data were set to 1. The A5 data are shown as a percent of the C5N data. **P<0.005. This experiment was done in duplicate with reproducible results.
4.4A. A5 cytoplasmic lysates were used to perform these mapping experiments because we did not observe HuR binding from C5N cytoplasmic fraction (Figure 4.2B).

The association of HuR protein with a synthetic ODC mRNA transcript was detected by Western blot analysis of the pulldown material (Figure 4.4B). Interestingly, fragments 1 and 2 (F1 and F2) exhibited even stronger HuR binding than the full length probe. Fragments 3 and 4 (F3 and F4) displayed lower levels of HuR binding, while the most distal fragment (F5) did not appear to associate with the HuR protein. The c-Myc full length 3’UTR positive control exhibited HuR binding and the GAPDH negative control demonstrated no binding (Liu, Rao et al. 2009). These data verify that HuR is able to bind to the more proximal region of the ODC 3’UTR, and suggest the presence of multiple binding sites.

To confirm these results and to further narrow the region of HuR binding, a second mapping experiment was performed using additional deletion constructs of either the proximal or distal end of the ODC 3’UTR. These deletion constructs ranged from 50 to 500 bases in length (Figure 4.5A). The full length ODC 3’UTR, deletion 1, deletion 2, and deletion 4 constructs all exhibited HuR binding, whereas deletion constructs 3 and 5 demonstrated little to no HuR binding (Figure 4.5B). Both sets of binding site data suggest that HuR binds to multiple sites on the 3’UTR of ODC between bases 1851 and 2151 (Figure 4.6A).

In order to verify that the region of HuR binding is between bases 1851 and 2151 on the ODC 3’UTR, a biotin pulldown was accomplished using a biotin-labeled probe spanning bases 1851 to 2151. The full length ODC 3’UTR was used a positive control and the deletion 5 construct was used as a negative control because it demonstrated no binding to the HuR protein, was approximately the same length as the 1851-2151 construct, and overlapped with the 1851-2151 construct at only 1 base. The 1851-2151 construct demonstrated binding to the HuR protein as did the full length ODC 3’UTR.
Figure 4.4. The HuR protein binds to the proximal region of the ODC 3’UTR.

A. Schematic of the overlapping biotin-labeled fragments used to map the HuR binding site. The overlapping fragments were made using primers and conditions described in the Materials and Methods chapter. The AUUUA ARE is located in the FL and F1 fragments. B. The proximal region of the ODC 3’UTR displays HuR binding. The overlapping biotin-labeled fragments were incubated with 120 µg of A5 cytoplasmic fraction to show the area(s) of HuR binding. The full length c-Myc 3’UTR (c-myc) was utilized as a positive control, the full length GAPDH 3’UTR (Gpdh) was used as a negative control, and the A5 whole-cell lysate (WC) was utilized as a positive control. HuR binding was measured by Western blot analysis of the pulldown material and GAPDH was used as a negative control to show pulldown specificity. This experiment was done in duplicate with reproducible results.
Figure 4.5. The HuR protein binds to a region on the ODC 3'UTR between bases 1851 and 2151.

A, A schematic of the truncation fragments used to map the region of HuR binding on the ODC 3'UTR. Truncations of the 5' end and 3' end of the ODC 3'UTR were used to map the HuR binding site. The AUUUA ARE is located in the Full 3'UTR, Deletion 1, and Deletion 2 truncations. B, A5 cytoplasmic fractions (120 µg) were used to map the HuR binding region on the ODC 3'UTR. A biotin pulldown assay was conducted as previously described in the Materials and Methods chapter. A Western blot of the pulldown material was used to measure HuR binding. A representative blot is shown in Panel B. This experiment was done in duplicate with reproducible results.
Figure 4.6. The region on the ODC 3’UTR between bases 1851 and 2151 demonstrates binding to the HuR protein.

A, The HuR binding site (1851-2151) is highlighted in gray. B, HuR binds to the region on the ODC 3’UTR between bases 1851 and 2151. Biotin-labeled probes for the full length ODC 3’UTR, the region between 1851 and 2151, and deletion 5 were created using primers and conditions described in the Materials and Methods chapter. The binding of HuR to the various biotin-labeled synthetic constructs was measured via Western blot analysis. The blot was stripped and reprobed for GAPDH to show binding specificity. A5 whole-cell lysate (WC) was used as a positive control. This experiment was done in duplicate with reproducible results.
The deletion 5 construct again displayed no binding to the HuR protein and the whole-cell lysate, another positive control, showed high levels of HuR (Figure 4.6B). These data support the mapping results shown in Figures 4.4 and 4.5 and again suggest that HuR binds between bases 1851 and 2151 on the ODC 3’UTR.

The HuR binding site data are interesting, as they demonstrate that HuR binds to multiple sites along the ODC 3’UTR. One potential issue with using the biotin-labeled synthetic transcripts to determine the exact binding site for HuR is that these constructs cause changes to the endogenous secondary structure of the ODC 3’UTR. It has been hypothesized that RNA binding proteins not only recognize sequence, but also recognize the secondary structure of an mRNA (Lopez de Silanes, Quesada et al. 2007). Thus, determining the HuR binding site via biotin-labeled synthetic transcripts could potentially be misleading as such truncations can either create or destroy RBP recognized secondary structures. To address this, m-fold software was used. M-fold allows one to insert an mRNA sequence into a database, and the software predicts the most energetically favorable mRNA secondary structure based on the sequence. The most energetically favorable secondary structure for each biotin-labeled probe is shown in Appendix A. The predicted ΔGs for each structure are shown in Table 4.1. There appears to be no consensus structure which HuR recognizes, as the predicted secondary structures of the full length 3’UTR, deletion 1, deletion 2, deletion 4, fragment 1, fragment 2, fragment 3, fragment 4, and the 1851-2151 base fragment are not similar. Furthermore, the ΔG values are quite different amongst the regions that bind HuR (Table 4.1) suggesting that the secondary structure of the ODC mRNA plays a minor role in determining HuR binding.
Table 4.1. ΔG values for each predicted m-fold RNA structure.

M-fold was used to create secondary structures for each region of the ODC 3'UTR used to map the HuR binding site. The most energetically favorable structure was chosen and the ΔG value was measured for each predicted RNA secondary structure.

<table>
<thead>
<tr>
<th>Structure</th>
<th>ΔG (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full ODC 3'UTR</td>
<td>-226.14</td>
</tr>
<tr>
<td>Deletion 1</td>
<td>-91.35</td>
</tr>
<tr>
<td>Deletion 2</td>
<td>-46.27</td>
</tr>
<tr>
<td>Deletion 3</td>
<td>-12.90</td>
</tr>
<tr>
<td>Deletion 4</td>
<td>-195.76</td>
</tr>
<tr>
<td>Deletion 5</td>
<td>-127.92</td>
</tr>
<tr>
<td>Fragment 1</td>
<td>-40.27</td>
</tr>
<tr>
<td>Fragment 2</td>
<td>-42.30</td>
</tr>
<tr>
<td>Fragment 3</td>
<td>-39.65</td>
</tr>
<tr>
<td>Fragment 4</td>
<td>-49.57</td>
</tr>
<tr>
<td>Fragment 5</td>
<td>-64.80</td>
</tr>
<tr>
<td>1851-2151 Fragment</td>
<td>-77.05</td>
</tr>
</tbody>
</table>
4.2.3  *HuR cellular localization differs between normal mouse keratinocytes and skin tumor-derived cells.*

The results above show strong binding of cytoplasmic HuR to ODC mRNA in transformed A5 cells, but little or no association in C5N keratinocytes, despite having similar levels of total HuR protein as seen in the whole-cell lysates of each cell line (Figure 4.2C). To address the issue of preferential binding of cytoplasmic HuR to the A5 ODC 3’UTR, the ODC 3’UTR was sequenced in both cell lines. An alignment of the two sequences revealed a 100 percent sequence identity (Figure 4.7). The sequence homology suggests that the difference in HuR binding to the ODC 3’UTR in A5 and C5N cells is not due to sequence or secondary structure changes. Given that the ODC 3’UTR was not mutated in the A5 cells, another possible explanation for our data is that the two cell lines display different cellular localizations of the HuR protein. HuR is predominately nuclear and has been shown to shuttle between the nucleus and cytoplasm in response to various stimuli or intrinsic cellular conditions. Cytoplasmic HuR in human tumors correlates with an increased tumor grade and a decreased survival rate (Blaxall, Dwyer-Nield et al. 2000; Heinonen, Bono et al. 2005). Based on this, we decided to investigate the cellular localization of the HuR protein in C5N and A5 cells.

The immunofluorescence results show that HuR is almost exclusively nuclear in C5N cells, whereas in A5 cells HuR is both nuclear and cytoplasmic (Figure 4.8). These data affirm our previous findings in Figures 4.2 and 4.3, as both the biotinylated pulldown assay and mRNP assay use cytoplasmic extract. The lack of cytoplasmic HuR presence in C5N cells accounts for HuR not binding to the ODC message in these cells. In fact, when C5N nuclear extracts were used to investigate HuR binding to the ODC mRNA transcript, we showed that HuR is able to bind to the ODC 3’UTR (Figure 4.9A).
Figure 4.7. The ODC 3’UTR sequence is identical in non-transformed C5N and transformed A5 cells.

cDNA for the ODC 3’UTR in C5N and A5 cells was made using the high fidelity PFU polymerase. The ODC 3’UTR for both cell lines was sequenced and the sequences were aligned with the *Mus musculus* (NM_013614) consensus sequence for the ODC 3’UTR using the MegAlign Software (DNASTAR, Madison, WI). Yellow boxes depict sequence regions that do not align with the *Mus musculus* sequence. The alignment was performed two independent times with reproducible results.
Figure 4.8. C5N keratinocytes display nuclear localization of the HuR protein and A5 spindle carcinomas display both nuclear and cytoplasmic localization of the HuR protein.

Cells for immunofluorescence were treated as described in the Materials and Methods chapter. HuR protein was detected using anti-HuR antibody (1:500). Cy-2 secondary antibody was used (1:200) (green fluorescence). The nucleus was stained using Dapi (1:1000) (blue fluorescence). Images were taken at a 40X magnification. Representative pictures are shown. This experiment was done in triplicate with reproducible results.
Figure 4.9. The C5N nuclear fraction binds to the ODC message.

A. Nuclear HuR in C5N cells binds to the ODC message. C5N nuclear extracts (120 µg) were incubated with biotin-labeled probes for the ODC 5'UTR (5'), coding region (CR), and 3'UTR (3'). Whole-cell lysate (WC) was used as a positive control. The biotin-labeled pulldown was accomplished according to the Materials and Methods chapter protocol. GAPDH was used to show any non-specific binding and to also confirm the presence of the whole-cell lysate. This experiment was done in triplicate with reproducible results. B. Quantification of the blot in Panel A. The blot was quantitated using Syngene software. All bands were normalized to a GAPDH control. The 5'UTR band quantitation was set to 1, and the coding region and 3'UTR band quantitations are reported as a percent of the 5'UTR band.
The C5N nuclear extract also displayed lower-level binding to the 5'UTR and coding region of the ODC mRNA transcript (Figure 4.9B). Mapping studies using C5N nuclear extracts and whole-cell lysates revealed that the HuR protein binds to multiple sites on the ODC 3'UTR. Similar to the mapping results in the A5 cells, C5N nuclear extracts exhibit binding to the ODC 3'UTR between bases 1851 and 2151 (Figures 4.10 and 4.11). Phosphorylation of the residues that reside within the HNS region of HuR has been shown to influence the localization of the protein (Doller, Huwiler et al. 2007; Doller, Akool el et al. 2008; Doller, Pfeilschifter et al. 2008). Thus, the immunofluorescence data shown here suggest that HuR is post-translationally modified at its HNS in A5 spindle carcinomas because we see an accumulation of HuR in the cytoplasm of these cells whereas in normal keratinocytes we see a predominately nuclear presence of the HuR protein.

4.2.4 *HuR silencing significantly decreases both ODC enzyme activity and the stability of ODC mRNA in A5 cells.*

To determine whether cytoplasmic HuR plays a causal role in increasing ODC enzyme activity and ODC mRNA stability in A5 cells, small interfering RNA (siRNA) against HuR was utilized to reduce intracellular HuR levels. A transient transfection of siHuR caused a 90 percent knockdown of HuR message as measured by qRT-PCR (Figure 4.12A). Moreover, the knockdown efficiency of the siHuR was assessed through Western blot analysis, and revealed a 70 percent decrease in HuR protein when compared to mock transfected and negative control siRNA treated cells (Figure 4.12B and 4.12C). To rule out toxicity, the cell viability of A5 cells treated with siHuR and the negative control siRNA was determined. No change in cell viability was measured in siHuR treated cells when compared to either untreated or negative control siRNA treated cells (Figure 4.13A). These data affirm that siHuR treatment in A5 cells is not toxic.
Figure 4.10. HuR binds to the proximal end of the ODC 3'UTR in C5N nuclear fractions.

A. Schematic of the ODC 3' UTR fragments used to map the HuR binding site. 150-200 base long overlapping fragments were used to map the binding site of HuR on the ODC 3' UTR using C5N nuclear fractions. The AUUUA ARE is located in the FL and F1 fragments. B. A biotin pulldown was conducted in C5N nuclear fractions according to the protocol described in the Materials and Methods chapter. HuR binding was assessed by Western blot analysis. GAPDH was used to show binding specificity. C5N whole-cell lysate (WC) and c-Myc (myc) were used as positive controls. This experiment was done in duplicate with reproducible results.
Figure 4.11. HuR binds between bases 1851 and 2151 on the ODC 3'UTR in C5N nuclear extracts.

A. Schematic of the truncation constructs used to map the HuR binding site(s) on the ODC 3'UTR. The AUUUA ARE is located in the Full length, Deletion 1, and Deletion 2 truncations. B. Biotin pulldowns were conducted as described in the Materials and Methods chapter using 120 µg of C5N nuclear extracts. HuR binding was assessed by Western blot analysis. GAPDH was used to show binding specificity. Whole-cell lysates (WC), the full length ODC 3'UTR (FL), and c-Myc (myc) were used as positive controls. The full length GAPDH 3'UTR (gdh) was used as a negative control. This experiment was done in duplicate with reproducible results.
Figure 4.12. siHuR does knockdown both HuR mRNA and protein in A5 spindle carcinoma cells.

A, HuR mRNA is knocked down in A5 spindle carcinoma cells. A5 cells were treated with siHuR or were mock transfected. After 72 h RNA was extracted according to the protocol described in the Materials and Methods chapter and HuR mRNA levels were assessed by qRT-PCR. The data were normalized to Cyclophilin A (n=3). Mock transfected cells were set to 100% and siHuR treated cells are shown as a percent of the mock. B, HuR protein level is significantly knocked down in A5 spindle carcinoma cells treated with siHuR. A5 cells were treated with siHuR, a negative control siRNA (Ctrl siRNA), or mock transfected. 72 h post-transfection, protein was harvested in 1X RIPA buffer. HuR level was measured via Western blot analysis. A5 whole-cell lysate (WC) was used as a positive control. GAPDH was used to show equal protein loading. This experiment was done in triplicate with reproducible results. C, Quantification of the Western blot from Panel B. Syngene software was used to quantitate the HuR and GAPDH bands. Each band quantitation is normalized to GAPDH. The whole-cell lysate band was set to 1 and each remaining band is a percent of the whole-cell lysate band.
Figure 4.13. siHuR does not alter cell viability but does decrease ODC enzyme activity and ODC mRNA stability in A5 spindle carcinoma cells.

A, Treatment of A5 cells with siHuR does not alter cell viability. Cell viability was measured in untreated, mock transfected, negative control siRNA (Ctrl siRNA), or siHuR as described in the Materials and Methods chapter. The OD measurements for untreated A5 cells were set to 1. The mock, Ctrl siRNA treated, and siHuR treated cells are reported as a mean percent of the untreated cells (n=9). B, ODC enzyme activity decreases in siHuR treated cells. 72 h post transfection, cells were extracted in ODC harvest buffer and ODC enzyme activities were measured as described in the Materials and Methods chapter. Mock values were set to 100% and siHuR and Ctrl siRNA values are shown as a percent of the mock. The values are means ± S.E. These experiments were done in triplicate with reproducible results (n=8). **P<0.005. C, ODC mRNA stability decreases in cells treated with siHuR. 72 h post transfection, A5 cells were treated with 11 µg of Actinomycin D and RNA was extracted at 0, 4, and 8 h. Northern blot analysis with qRT-PCR verification was used to determine the ODC mRNA half-life in Ctrl siRNA treated cells and qRT-PCR was used for siHuR treated cells. ODC mRNA levels were normalized to Cyclophilin A at each time point. The values are means ± S.E. (n=3-9). ODC mRNA half-life was determined using exponential curve fit analysis.
To measure the level of ODC protein after the addition of siHuR to the A5 cells, we used an ODC enzyme activity assay, since ODC activity correlates with protein levels. On average, a 60 percent decline in ODC enzyme activity occurred with the addition of siHuR. Conversely, the control siRNA had no effect on the ODC enzyme activity in the A5 cells (Figure 4.13B). In addition, the ODC mRNA stability decreased by approximately 60 percent when A5 cells were treated with siHuR compared to cells that had been treated with the negative control siRNA (Figure 4.13C). These findings strongly suggest that HuR enhances the stability of the ODC mRNA transcript in mouse spindle carcinoma cells by directly binding to the ODC 3’UTR, and that this increased stability contributes to the observed induction of ODC enzyme activity in these cells.

To ensure that the knockdown of cytoplasmic HuR played a causal role in the reduction of both ODC enzyme activity and ODC mRNA stability in A5 cells, we assessed the effects of knocking down HuR in C5N cells. Since C5N express HuR exclusively in the nucleus (Figure 4.8), we would not expect HuR knockdown in these cells to affect ODC mRNA stability. Treatment of C5N cells with siHuR resulted in a decrease in whole-cell HuR levels (Figure 4.14A). In fact, HuR protein levels decreased by approximately 50% in siHuR treated cells when compared to cells that had been treated with the negative control siRNA (Figure 4.14B). However, the knockdown of HuR had no effect on the ODC mRNA stability in these cells. Both siHuR treated and negative control siRNA treated cells had the same ODC mRNA half-life of approximately 9 h (Figure 4.14C).

The ODC mRNA half-life in C5N cells that had been treated with either the siHuR or the negative control siRNA was approximately 2-fold longer than untreated C5N cells. The use of the lipid transfection reagent or the siRNA duplexes may explain this result. In fact, we saw a stabilization of ODC mRNA in A5 cells treated with the same negative control siRNA (compare Figure 3.5 to 4.13C). In any case, we show that
Figure 4.14. Treatment of C5N keratinocytes with siHuR does not alter ODC mRNA stability.

A, HuR protein is significantly knocked down in C5N cells treated with siHuR. 72 h post transfection, protein was harvested in 1X RIPA buffer. Equal amounts of protein were loaded onto a 10% SDS-PAGE gel and Western blot analysis was conducted for HuR and GAPDH. Negative control siRNA (Ctrl siRNA) was used as a control. B, Quantification of the Western blot from Panel A. Syngene software was used to quantitate the HuR and GAPDH bands. HuR bands are normalized to GAPDH. The Ctrl siRNA band was set to 1 and the siHuR quantitation is shown as a percent of the control. C, ODC mRNA stability does not change in C5N cells treated with siHuR. 72 h post-transfection, cells were treated with 11 µg of Actinomycin D and RNA was extracted at 0, 4, and 8 h. qRT-PCR was used to measure the levels of ODC and Cyclophilin A mRNA. ODC mRNA levels were normalized to Cyclophilin A at each time point. The values are the means ± S.E. (n=6). ODC mRNA half-life was determined using exponential curve fit analysis.
ODC mRNA stability is unchanged in C5N cells treated with siHuR and that A5 cells treated with siHuR demonstrate a reduction in both ODC enzyme activity and ODC mRNA stability. These data support our hypothesis that the cytoplasmic accumulation of HuR in NMSC progression is at least, in part, responsible for the observed increases in ODC mRNA stability and ODC enzyme activity.

4.2.5 The cytoplasmic accumulation of HuR in A5 spindle carcinoma cells influences HuR binding to other mRNA transcripts associated with tumorigenesis.

HuR has been shown to increase the mRNA stability of various transcripts. During neoplastic transformation, HuR has been shown to bind to and stabilize mRNAs that are associated with neoplastic transformation such as c-Myc, COX-2, and ProTα (Lal, Kawai et al. 2005; Mazan-Mamczarz, Hagner et al. 2008; Zhang and Bowden 2008; Liu, Rao et al. 2009). Thus, we decided to investigate whether the cytoplasmic accumulation of HuR caused an increase in the mRNA stability of other transcripts associated with carcinogenesis. We specifically looked at the binding of HuR to the COX-2, TNF-α, IL-8, and VEGF mRNAs because HuR has been shown to associate with these transcripts in other model systems and because these transcripts are associated with neoplasia (Dean, Wait et al. 2001; Dixon, Tolley et al. 2001; Nabors, Gillespie et al. 2001; Erkinheimo, Lassus et al. 2003; Zhang and Bowden 2008). A5 cytoplasmic extracts demonstrated HuR binding to all four mRNA 3'UTRs. Not surprisingly, C5N cytoplasmic extracts displayed no HuR binding to the 3'UTRs of COX-2, TNF-α, IL-8, or VEGF (Figure 4.15). To assess the effect of HuR binding to these transcripts the mRNA stability of COX-2 and VEGF was determined in both the C5N and A5 cells. Unlike ODC, the VEGF mRNA was not stabilized in A5 spindle carcinoma cells when compared to C5N keratinocytes. In fact, the VEGF mRNA decayed very rapidly with the same half-life in both cell lines (Figure 4.16). These data demonstrate the complexity of the mechanisms behind the control of mRNA stability, which usually involves the association
Figure 4.15. Other mRNA transcripts associated with neoplastic transformation associate with the HuR protein in A5 cytoplasmic extracts but not in C5N cytoplasmic extracts.

A5 and C5N cytoplasmic fractions were incubated with the full length 3'UTR biotin-labeled probes for COX-2, TNF-α, IL-8, and VEGF. The ODC 3'UTR was used as a positive control. A biotin pulldown was conducted as described in the Materials and Methods chapter. The synthetic mRNA probes were tested for HuR binding by Western blot analysis. Each blot was stripped for GAPDH to assess non-specific binding. This experiment was done in duplicate with reproducible results.
Figure 4.16. VEGF mRNA stability is unchanged between A5 spindle carcinoma cells and C5N keratinocytes.

Cells were treated with 11 µg of Actinomycin D upon 70% confluency, and RNA was extracted at 0, 4, and 8 h. qRT-PCR was used to measure the levels of VEGF and Cyclophilin A mRNA. VEGF mRNA levels were normalized to Cyclophilin A at each time point and the mRNA half-life was determined by curve fit analysis. The values are the means ± S.E. (n=6). This experiment was done in duplicate with reproducible results.
of multiple RBPs. The level of COX-2 mRNA in both cell lines was below the level of
detection (data not shown), suggesting that COX-2 mRNA is not abundant in these cells.
Together, these data show that cytoplasmic HuR is able to bind to the 3'UTRs of other
transcripts, and does not specifically bind to ODC. In addition, these data reveal that
HuR binding does not always correlate with an increase in mRNA stability as VEGF
mRNA stability was unchanged in A5 spindle carcinoma cells.

4.2.6 Studies to determine an upstream mechanism that regulates HuR shuttling.

Numerous proteins and pathways, including PKC and AMPK, have been shown
to regulate HuR nucleocytoplasmic shuttling [Reviewed in (Doller, Pfeilschifter et al.
2008)]. For example, HuR is exported out of the nucleus through its association with
transportin 1 (Trn1) and Trn2 (Guttinger, Muhlhausser et al. 2004). Moreover, HuR has
been shown to associate with the nuclear ligand pp32, a nuclear phosphoprotein, and
APRIL, a member of the TNF family associated with apoptosis, which contain nuclear
export signals that are recognized by the CRM1 export receptor (Gallouzi and Steitz
2001). Although a few signaling pathways have been shown to post-translationally
modify HuR and cause its sequestration in either the cytoplasm or nucleus, more
upstream targets need to be elucidated (Li, Park et al. 2002; Doller, Pfeilschifter et al.

The mammalian target of Rapamycin (mTOR) pathway is an attractive candidate
for regulating HuR localization because AMPK, which is upstream of mTOR, has
previously been suggested to regulate HuR shuttling in human colorectal carcinoma
cells, RKO cells, and rat intestinal epithelial cells, IEC-6 cells (Wang, Fan et al. 2002;
Wang, Yang et al. 2004; Zou, Liu et al. 2008). Moreover, the addition of the mTORC1
inhibitor, Rapamycin, was shown to decrease VEGF mRNA stability in two renal cancer
cell lines (786-O and Cak-1 cells), suggesting that mTOR and RBPs may play a role in
mediating the mRNA stability of labile mRNA transcripts (Basu, Datta et al. 2010).
mTOR is a member of the phosphoinositide-3-kinase-related kinase family, which are highly conserved from yeast to humans. The mTORC1 complex contains the proteins mTOR, raptor, and LST8. This complex regulates protein synthesis and is sensitive to Rapamycin treatment. The mTORC2 complex consists of the proteins mTOR, Rictor, LST8 and sin1, and is insensitive to Rapamycin treatment.

The kinase activity of mTORC1 is stimulated by the GTP-bound form of Ras homolog enriched in the brain (RHEB). RHEB is regulated by the tumor suppressor heterodimer composed of tuberous sclerosis 1 (TSC1) and TSC2. The main downstream targets of mTORC1 are 4E-BP and the p70 S6 kinase, which both activate translation machinery [Reviewed in (Gibbons, Abraham et al. 2009; Ma and Blenis 2009)].

Previously, ODC enzyme activity has been shown to be reduced in Ras activated RIE-1 cells that had been treated with the mTORC1 specific inhibitor Rapamycin (Origanti and Shantz 2007). Thus, based on the aforementioned data, we decided to investigate the effect of Rapamycin on HuR shuttling, ODC mRNA stability, and ODC enzyme activity in A5 spindle carcinoma cells. A5 cells treated with 100 nM Rapamycin showed a decrease in ODC enzyme activity at 8 and 10 h when compared to DMSO treated A5 cells (Figure 4.17). However, ODC steady-state mRNA levels did not change in the presence of Rapamycin (Figure 4.18). To determine if mTORC1 inhibition with Rapamycin affected ODC mRNA stability, we first investigated the localization of HuR in A5 cells treated with Rapamycin. We hypothesized that the addition of Rapamycin would cause the nuclear accumulation of HuR in A5 spindle carcinoma cells, which would lead to a decrease in both the ODC mRNA stability and ODC enzyme activity. Examination of the immunofluorescence revealed variable results, with no consistent change in the localization of the HuR protein in the presence of Rapamycin (Figure 4.19). Furthermore, Northern blot analysis of A5 mRNA showed that the half-life of ODC
**Figure 4.17.** Rapamycin treatment causes a decrease in ODC enzyme activity at 8 and 10 h in A5 spindle carcinoma cells.

A5 cells were treated with 100 nM Rapamycin as described in the Materials and Methods chapter. At 3, 6, 8, 10, and 24 h post Rapamycin treatment, A5 cells were harvested in ODC harvest buffer. ODC enzyme activity was determined as previously described. This experiment was done in duplicate with reproducible results. \*P<0.05.
Figure 4.18. Rapamycin treatment does not change the ODC steady-state mRNA level in A5 spindle carcinoma cells.

A5 cells were treated with 100 nM Rapamycin as described in the Materials and Methods chapter. At 2, 8, 12, and 24 h post Rapamycin treatment, RNA was extracted and qRT-PCR was conducted in order to determine the steady-state level of ODC mRNA. ODC mRNA was normalized to Cyclophilin A mRNA at each time point. This experiment was done in duplicate with reproducible results.
Figure 4.19. Rapamycin treatment does not alter the cellular localization of the HuR protein.

A5 spindle carcinoma cells were seeded onto poly-L-lysine coated glass coverslips and upon 70% confluency, cells were treated with Rapamycin (100 nM) for 1, 4, 8 or 24 h. At each time point the cells were fixed and treated for HuR immunofluorescence according to the Materials and Methods chapter.
was unchanged in A5 cells treated with Rapamycin (data not shown). These data suggest that although mTORC1 appears to play a role in determining the ODC protein level, this complex is not acting via HuR or an mRNA stability mechanism in this model. The data suggest that mTORC1 may be affecting the ODC translation efficiency.

AMPK is an enzyme that participates in the cellular response to metabolic stress. Its activity is strongly elevated in response to a high cellular AMP:ATP ratio. Depletion of growth factors or glucose as well as treatment with the pharmacological agent 5-amino-imidazole-4-carboxamide riboside (AICAR), which mimics the effects of AMP, causes AMPK to be activated. Once activated, AMPK phosphorylates metabolic enzymes causing the global inhibition of biosynthetic pathways in order to conserve energy and induces catabolic pathways in order to produce more ATP [Reviewed in (Corton, Gillespie et al. 1995; Motoshima, Goldstein et al. 2006)].

A decrease in AMPK activity was associated with the cytoplasmic accumulation of HuR as mentioned above. Treatment with AICAR can cause either a decrease or an increase in the levels of cytoplasmic HuR. This is dependent on the cell line used (Wang, Fan et al. 2002; Wang, Yang et al. 2004; Martinez-Chantar, Vazquez-Chantada et al. 2006). Based on these results we treated both the C5N keratinocytes and A5 spindle carcinoma cells with AICAR. No change in HuR cellular localization was observed in either cell line at 4 h (Figure 4.20). The 4 h time point was used because previous studies had found HuR shuttling out of the cytoplasm and into the nucleus between 1 and 6 h after AICAR treatment (Wang, Fan et al. 2002). These data suggest that AMPK is not responsible for the cellular distribution of HuR in our cell model.

PKC, specifically the classical α and novel δ isoforms have been implicated in the cytoplasmic localization of HuR. Doller et al. showed that PKCα-dependent phosphorylation of HuR was critical for ATP-induced HuR cytoplasmic accumulation in mesangial cells (Doller, Huwiler et al. 2007). Moreover, in neuroblastoma cells, the
Figure 4.20. Treatment of C5N and A5 cells with the AMPK activator AICAR does not alter the localization of HuR.

C5N and A5 cells were seeded onto glass coverslips that had been pre-coated with poly-L-lysine and upon 70% confluency, the cells were serum starved for 24 h. Cells were treated with 2 mM or 4 mM of AICAR and the cells were fixed 4 h after treatment according to the protocol described in the Materials and Methods chapter. HuR immunofluorescence was accomplished using the previously described protocol.
PKCα-induced shuttling of HuB, HuC, and HuD, as a result of the application of phorbol esters, enhanced the stability of growth-associated protein 43 (GAP-43) (Pascale, Amadio et al. 2005). Angiotensin II (AngII) induced the PKCδ-dependent phosphorylation of HuR at serines 221 and 318. The phosphorylation of HuR by PKCδ caused the cytoplasmic accumulation of the HuR protein and the subsequent stabilization of COX-2 in mesangial cells (Doller, Akool el et al. 2008).

Previous data have shown that PKC α and δ activation correlates with HuR cytoplasmic accumulation. Furthermore, PKC α is known to be activated by the phorbol ester TPA (Pascale, Amadio et al. 2005). Based on these data, we decided to treat the C5N keratinocytes with TPA to try to cause HuR cytoplasmic accumulation in these cells. In human U937 lymphoma cells, it was shown that HuR localized to the cytoplasm 5 h after application of 16 nM TPA (Bandyopadhyay, Sengupta et al. 2008). In C5N cells, TPA did not consistently change the cellular localization of HuR at 1, 4, 6, or 8 h after application suggesting that the keratinocyte lineage behaves differently than transformed lymphoma cells and mesangial cells to TPA treatment (Figure 4.21).

4.3 Discussion

The data described here show for the first time that the rate-limiting enzyme in the polyamine biosynthetic pathway, ornithine decarboxylase (ODC), is regulated by binding of its mRNA to the stabilizing RNA binding protein HuR. These data are novel, as ODC mRNA has never before been shown to be regulated by such a cis- and trans-acting mechanism. Our results demonstrate that binding of the trans-acting factor HuR increases ODC enzyme activity and mRNA stability via cis-acting elements on the 3'UTR of the ODC mRNA transcript (Figures 4.2, 4.4, 4.5). Data from deletion constructs and overlapping fragments indicate that HuR may bind to multiple sites within the ODC 3'UTR. The biotin-pulldowns conducted in these experiments are
Figure 4.21. Application of C5N cells with 15.4 nM TPA does not induce the cytoplasmic accumulation of HuR.

C5N cells were seeded onto glass coverslips that had been pre-coated with poly-L-lysine. Upon 70% confluency, cells were treated with TPA for 1, 4, 6, or 8 h. At each time point the cells were fixed for HuR immunofluorescence, in order to determine the cellular localization of the HuR protein, according to the Materials and Methods chapter.
semi-quantitative and provide essential information about ODC mRNA regulation by HuR. Demonstrating binding of HuR to the ODC synthetic transcripts is in and of itself important and novel. One caveat of using the biotin-labeled synthetic transcript method for determining the binding site of HuR on the ODC 3'UTR is that the use of any construct other than the full length 3'UTR of ODC creates a different predicted secondary structure and thus makes it difficult to determine whether HuR recognizes the sequence or structure of the synthetic transcript. To circumvent these issues a few different approaches to mapping the binding site of HuR on the ODC 3'UTR can be utilized. RIP-ChIP is a procedure in which the RNA is cross-linked to the HuR protein, the HuR protein is immunoprecipitated, and the location of HuR binding along the mRNA is identified. This method could more closely map the HuR binding site without manipulating the endogenous secondary structure of the mRNA. Another possible procedure to more accurately map the HuR binding site on the ODC 3'UTR is to use a RNA EMSA (REMSA). However, by using this method you are limited to a 50-100 nucleotide RNA fragment, which would again alter secondary structure of the RNA, and would require a purified HuR protein. Although our method for mapping the HuR binding site on the ODC 3'UTR has drawbacks, it is an accepted method in the field of RBP biology for mapping RBP binding sites, and it provides a clear readout as to whether a RBP is, or is not, binding to a given fragment of the mRNA’s 3’UTR.

While the dogma is that HuR binds to classical AREs, the exact binding motif for HuR remains unclear. Lopez de Silanes et al. have shown that HuR binds to U-rich elements (Lopez de Silanes, Zhan et al. 2004). Furthermore, Yeap et al. demonstrated HuR binding to UC-rich motifs on the 3'UTR of androgen receptor mRNA (Yeap, Voon et al. 2002). The general area of strongest HuR binding on the ODC 3'UTR, between bases 1851 and 2151, is also U-rich (Figure 4.6).
The results presented here show that cytoplasmic HuR binds to the ODC 3’UTR of A5 spindle carcinoma cells, but no association was demonstrated in C5N keratinocytes (Figure 4.2B and Figure 4.3). The stabilizing affect of HuR on the ODC 3’UTR corresponds to the localization of the HuR protein, as HuR was cytoplasmic in A5 cells but was strictly nuclear in C5N cells (Figure 4.8). This phenomenon is in accordance with previous results showing that the cytoplasmic localization of HuR stabilizes p53 and nucleophosmin (Zou, Mazan-Mamczarz et al. 2006). Additionally, in studies investigating the role of HuR in human tumor samples, a strong correlation was found between tumor grade and HuR cytoplasmic accumulation in serous ovarian carcinoma, Merkel cell carcinoma, and human invasive ductal breast carcinoma. Non-neoplastic tissue controls exhibited little cytoplasmic HuR presence (Erkinheimo, Lassus et al. 2003; Heinonen, Bono et al. 2005; Koljonen, Bohling et al. 2008). C5N nuclear extracts, which have an abundance of HuR protein, demonstrate binding to a synthetic ODC 3’UTR transcript (Figures 4.9-4.11). Furthermore, C5N nuclear HuR exhibits binding to the same region on the ODC 3’UTR as the A5 cytoplasmic HuR (Figures 4.10 and 4.11). Given that there are no mutations in the 3’UTR of ODC, these data also suggest that HuR localization dictates whether the HuR protein binds to and stabilizes the ODC transcript (Figure 4.7).

It has been found previously, using a rat intestinal epithelial cell model, that polyamine depletion can cause a decrease in AMPK activity and thereby cause the retrograde movement of HuR from the nucleus into the cytoplasm via importin α1 (Zou, Liu et al. 2008). Furthermore, the depletion of the polyamine intracellular pool through the down-regulation of ODC was shown to cause an increase in HuR cytoplasmic presence and an increase in ATF-2 mRNA stability (Xiao, Rao et al. 2007). In contrast, we do not see HuR shuttle from the nucleus in response to polyamine depletion in our NMSC model. In fact, we see the opposite in our cells, since A5 carcinoma cells, where
HuR is present in the cytoplasm, contain higher putrescine levels when compared to non-transformed C5N keratinocytes (Table 3.2), which contain measureable HuR only in the nucleus. While the reason for this difference is not known, it is likely that other factors in addition to the polyamines play a role in controlling HuR shuttling. For example, HuR can shuttle from the nucleus to the cytoplasm during cell cycle progression or after UVB exposure (Atasoy, Watson et al. 1998; Kim and Gorospe 2008; Zhang and Bowden 2008). AMPK activation with AICAR also has no effect on HuR localization in our model, suggesting keratinocytes respond differently than intestinal-derived cells and RKO cells.

We note in A5 spindle carcinoma cells that both ODC enzyme activity and ODC mRNA half-life are upregulated (Figures 3.2 and 3.5). The increase in ODC mRNA stability in A5 cells is due, at least in part, to the association of cytoplasmic HuR with the ODC transcript. Given that a mutated HuR protein has never been reported in cancer, our results suggest the possibility that HuR is post-translationally modified at the HNS in A5 cells which causes its cytoplasmic accumulation and allows it to bind to a variety of target mRNAs, including ODC (Lopez de Silanes, Lal et al. 2005; Doller, Huwiler et al. 2007; Doller, Akool el al. 2008). Because upstream regulators of HuR localization are still being elucidated, we decided to investigate three candidates which could control HuR shuttling. Similar to our AICAR experiments, treatment with Rapamycin or TPA displayed variable results and showed no reproducible affect on HuR localization in A5 or C5N cells respectively. The data from these experiments, although suggesting that these pathways are not implicated in HuR shuttling in our cell model of NMSC, are preliminary. To completely rule out these candidate pathways, more comprehensive dose responses and time courses will need to be carried out. However, based on our data, an upstream regulator of HuR shuttling in the murine NMSC model still needs to be elucidated. To date, several post-translational modifications to the HuR protein have
been shown to influence the localization of HuR. For example, an S242A mutant of HuR accumulates in the cytoplasm and also increases its ability to bind to its targets Cyclin A2 and B1 in HeLa cells (Kim, Yang et al. 2008). Future experiments will determine whether post-translational modifications of HuR alter its cellular localization in our mouse NMSC model.

Finally, we have demonstrated that cytoplasmic HuR not only binds to the ODC mRNA transcript, but is also capable of binding to other transcripts such as COX-2, IL-8, TNF-α, and VEGF (Figure 4.15). Interestingly, HuR binding does not always correlate with an increase in mRNA stability, as revealed by the VEGF mRNA stability data (Figure 4.16). However, our data clearly show that the ODC transcript is stabilized by HuR, as undoubtedly are others, in NMSC development.

It is well established that NMSC carries both a health and fiscal burden, costing approximately $1.5 billion to treat in the United States alone in 2004 [Reviewed in (Bickers, Lim et al. 2006)]. ODC has been shown to be both necessary and sufficient for the onset of NMSC in mice, and is also elevated in human NMSC (O'Brien, Megosh et al. 1997; Smith, Trempus et al. 1998; Feith, Shantz et al. 2001; Gilmour 2007; Elmets and Athar 2010). Thus, the link between ODC and NMSC is indisputable, validating ODC as both a chemoprevention and chemotherapeutic target. In fact, a recent phase III chemoprevention trial demonstrated that DFMO, a suicide inactivator of ODC, markedly reduces the incidence of new basal cell carcinoma in subjects with a history of skin cancer (Bailey 2010). Earlier studies showed that topical DFMO administration reduced the incidence of actinic keratosis, a precursor of squamous cell carcinoma, in subjects who are high risk for NMSC (Einspahr, Nelson et al. 2002). The studies provided here shed more light into the mechanism of ODC regulation in the skin and can provide an additional target for the development of chemopreventive and chemotherapeutic agents that alter ODC activity.
In summary, we describe a *cis*- and *trans*- acting mechanism regulating ODC mRNA stability and ODC enzyme activity in the skin. We demonstrate, using a murine model of NMSC, that ODC activity is increased and mRNA is stabilized in carcinomas, and that the stabilization of the ODC transcript is due to the binding of HuR to the ODC 3’UTR. HuR is localized to the cytoplasm in spindle carcinoma cells whereas in normal keratinocytes it is exclusively nuclear. This alteration in the localization of the HuR protein accounts for the discrepancy of HuR binding to the ODC 3’UTR between the two cell lines. Furthermore, we provide a functional link between HuR and ODC using RNAi to knockdown HuR. HuR knockdown resulted in the destabilization of the ODC message and a decrease in ODC activity. These findings not only demonstrate a novel mechanism of ODC regulation, but also provide another potential target for chemoprevention and chemotherapeutics for NMSC.
CHAPTER 5

Studies on ODC regulation by the RNA binding protein Tristetraprolin (TTP)

5.1 Introduction

In Chapter 4 we demonstrated that ODC is regulated at the level of mRNA stability in keratinocyte-derived cells by binding to cytoplasmic HuR. HuR, a stabilizing RBP, is known to bind to mRNA transcripts that are stabilized during carcinogenesis such as COX-2 and ProTα (Lal, Kawai et al. 2005; Mazan-Mamczarz, Hagner et al. 2008; Zhang and Bowden 2008). Moreover, the cytoplasmic accumulation of HuR is associated with high tumor grade and poor disease prognosis in numerous cancers (Lopez de Silanes, Fan et al. 2003; Heinonen, Bono et al. 2005; Koljonen, Bohling et al. 2008). Interestingly, other work in our lab has shown that another RBP binds to the ODC 3'UTR in rat intestinal epithelial (RIE-1) cells (Origanti and Shantz, unpublished data). Specifically, we have shown that the RBP tristetraprolin (TTP) binds to the ODC 3'UTR in both untransformed RIE-1 cells and also in RIE-1 cells expressing an activated mutant of Ha-Ras (Ras12V cells). Thus, we decided to investigate the level of TTP binding to the ODC 3'UTR in C5N keratinocytes and A5 spindle carcinoma cells. We predict that TTP will bind more strongly to the ODC 3'UTR in C5N cells when compared to A5 cells, since TTP generally destabilizes its target mRNA (Lai, Carballo et al. 1999; Carballo, Lai et al. 2000; Stoecklin, Ming et al. 2000; Eberhardt, Doller et al. 2007).

TTP is one of three widely distributed RNA binding proteins which contain Cys-Cys-His-zinc (CCCH-zinc) finger RNA-binding domains. Other family members of TTP include: butyrate response factor 1 (BRF1) and BRF2 [Reviewed in (Kulkarni, Ozgur et al. 2010)] These RBPs classically bind to UUAUUUAUUU sequences (Brewer, Malicka et al. 2004). TTP binds to, and destabilizes, numerous transcripts such as TNF-α, GM-CSF, VEGF, and IL-3 (Lai, Carballo et al. 1999; Carballo, Lai et al. 2000;
Stoecklin, Ming et al. 2000; Suswam, Li et al. 2008; Lee, Son et al. 2009). Moreover, TTP interacts with proteins that are associated with RNA decay such as DCP1, DCP2, XRN1, and CCR4, and has been shown to stimulate mRNA deadenylation and promote mRNA decay (Lai, Carballo et al. 2000; Fenger-Gron, Fillman et al. 2005; Lykke-Andersen and Wagner 2005). Similar to HuR, TTP has been found to shuttle rapidly from the nucleus to the cytoplasm in fibroblasts in response to serum and phorbol esters (Taylor, Thompson et al. 1996). In response to cellular stress, TTP has been shown to localize to stress granules and P-bodies and it is hypothesized that this is to relocate stalled mRNAs to the mRNA decay machinery (Stoecklin, Stubbs et al. 2004; Kedersha, Stoecklin et al. 2005).

Studies show that TTP expression is lower in tumor samples when compared to non-tumorigenic tissue samples. In an array study using a variety of epithelial cancer cDNA samples, as well as non-transformed controls, TTP mRNA levels were shown to be decreased in 65% of tumors when compared to non-transformed controls. The reduction in TTP mRNA was frequent in thyroid, breast, lung, ovary, and uterine tumors. Western blot analysis confirmed that TTP protein expression was also suppressed in tumor samples (Brennan, Kuwano et al. 2009). These data suggest that TTP expression is frequently repressed in epithelial tumor samples, and correlates TTP expression with tumor development.

A common model used for studying TTP is a mouse embryonic fibroblast cell line created by the Blackshear group in which the TTP protein is truncated and non-functional (Taylor, Carballo et al. 1996). The MEFs isolated from these mice were harvested at E14.5. The TTP knockout mice are phenotypically normal at birth, but soon after start to exhibit low body weight, arthritis, dermatitis, and conjunctivitis. Moreover, these mice display lifespans of 7 to 16 months.
Based on previous results observed in our lab, our main goal was to characterize the relationship between TTP and the ODC mRNA transcript in mouse keratinocyte-derived cell lines. The data described here reveal that cytoplasmic TTP binds to the ODC 3′UTR in spindle carcinoma cells. However, only nuclear TTP binds to the ODC 3′UTR in non-transformed keratinocytes. ODC mRNA stability is enhanced in TTP knockout MEFs when compared to TTP wild-type MEFs, but the ODC enzyme activity is similar between the two cell lines. Moreover, we show that the localization of the HuR protein is unchanged in these cells.

5.2 Results

5.2.1 Cytoplasmic TTP binds to the ODC 3′UTR in spindle carcinoma cells and nuclear TTP binds to the ODC 3′UTR in non-transformed keratinocytes.

In order to assess the relationship between TTP and ODC mRNA stability, we investigated whether TTP was able to bind to the ODC 3′UTR in C5N and/or A5 cells. We have previously shown that TTP can bind to the ODC 3′UTR in RIE-1 cells (unpublished data), and therefore hypothesized that both cell lines would exhibit TTP binding to the ODC message. A pulldown was conducted in which synthetic, biotin-labeled ODC 3′UTR, coding region (CR), and 5′UTR probes were used. The binding of cytoplasmic TTP to the ODC mRNA was measured by Western blot analysis of the pulldown material. Cytoplasmic TTP exhibited binding to the 5′UTR, CR, and 3′UTR of ODC in A5 spindle carcinoma cells. Surprisingly, cytoplasmic TTP binds to only the CR in C5N keratinocytes (Figure 5.1). Therefore, we wanted to determine whether nuclear TTP was able to bind to the ODC mRNA transcript in C5N cells. Western blot analysis revealed that nuclear TTP in C5N cells was able to bind strongly to the CR and 3′UTR of
Figure 5.1. Cytoplasmic TTP binds to the 5'UTR, coding region, and 3'UTR of ODC in A5 spindle carcinoma cells but only binds to the ODC coding region in C5N cells.

C5N and A5 cytoplasmic extracts were used in a biotin reaction in which 15 µl of biotin-labeled ODC 3'UTR, 5'UTR, or coding region (CR) was incubated with 120 µg of cytoplasmic extract. The pulldown material was assessed for TTP by Western blot analysis. Verification of cytoplasmic purity was determined by HuR binding, as A5 cells displayed typical HuR binding whereas no HuR binding was exhibited in C5N cytoplasmic extracts (data not shown). This experiment was done once and needs to be repeated in order to validate the results.
ODC with less but measurable binding to the 5’UTR (Figure 5.2). These data can be interpreted two ways since we know the ODC 3’UTR sequence is the same in both cell lines. First, cytoplasmic TTP is post-translationally modified in A5 cells, causing TTP to bind to the UTRs and CR of ODC. Second, nuclear TTP in C5N cells contain post-translational modifications which cause it to bind indiscriminately to the entire ODC mRNA transcript, a trend we also see with A5 cytoplasmic TTP (Compare Figures 5.1 and 5.2).

Based on observations by Brennan et al., which found that TTP expression was repressed in various types of cancer, we hypothesized that total TTP protein levels would be reduced in transformed A5 cells when compared to non-transformed C5N cells (Brennan, Kuwano et al. 2009). However, we saw similar levels of TTP between C5N and A5 cells (Figure 5.3). A common trend was the presence of a TTP doublet in C5N cells. The doublet may be non-specific binding of the antibody. Alternatively, the doublet may represent a post-translationally modified species of TTP in C5N cells, whereas A5 cells contain one species of the protein. TTP post-translational modifications are typical as the size of TTP ranges from 37 kD to 50 kD due to phosphorylation. Upon further examination of TTP using A5 and C5N cellular fractionations, we saw that there was a change in the banding pattern of TTP in C5N nuclear and cytoplasmic extracts. A similar banding pattern for TTP was observed in A5 nuclear and cytoplasmic fractions (Figure 5.4). In fact, both A5 cytoplasmic and nuclear fractions had a similar TTP banding pattern to the C5N nuclear extract. These data suggest that TTP may be post-translationally modified in C5N cytoplasmic extracts in such a way that makes the protein unable to bind to the UTRs of ODC, but that allow it to bind to the ODC CR.
Figure 5.2. Nuclear TTP binds to the ODC mRNA transcript in C5N cells.

C5N nuclear extracts were used in a biotin reaction in which 15 µl of biotin-labeled ODC 3'UTR (3'), 5'UTR (5'), or coding region (CR) was incubated with 120 µg of nuclear extract. The pulldown material was assessed for TTP binding by Western blot analysis. Verification of the nuclear purity was determined by HuR binding, as C5N cells displayed typical HuR binding in this experiment (data not shown). This experiment was done once and needs to be repeated in order to validate the results.
Figure 5.3. Whole-cell TTP protein levels are similar between C5N and A5 cells.

A, C5N and A5 whole-cell lysates display equal amounts of TTP protein. 1X RIPA buffer was used to extract total cellular protein. TTP levels in C5N and A5 whole-cell extracts were assessed by Western blot analysis. Equal loading was measured with GAPDH. This experiment was done in duplicate with reproducible results. B, Quantitation of the blot from Panel A. Bands were quantitated using Syngene Software. TTP bands were normalized to GAPDH.
Figure 5.4. C5N cytoplasmic TTP displays a different banding pattern than A5 nuclear, A5 cytoplasmic, and C5N nuclear TTP.

C5N and A5 cells were fractionated as described in the Materials and Methods chapter. The extracts had previously been tested for fractionation purity (data not shown). The fractionation samples for each cell line were run on a 10% SDS-PAGE gel and Western blot analysis was conducted for TTP. Three separate nuclear and cytoplasmic fractions were run for A5 cells and all produced similar results. Three separate nuclear and one cytoplasmic C5N fraction were run and produced similar results.
5.2.2 The TTP protein binds to multiple sites on the ODC 3'UTR in both C5N and A5 cells.

To map the TTP binding site(s) on the ODC 3'UTR, 150 base long overlapping fragments were used that scanned the entire length of the ODC 3'UTR (Refer to Figure 4.4). These fragments were labeled with biotin, and pulldown assays were conducted as previously described. Using cytoplasmic lysates of A5 cells, we observed that TTP binds to fragments 2 and 5 (F2 and F5) on the ODC 3'UTR (Figure 5.5). Nuclear lysates of C5N cells showed that TTP binds to each fragment along the ODC 3'UTR (Figure 5.6). The difference in TTP binding sites between A5 cytoplasmic fractions and C5N nuclear fractions may be due to different post-translational modifications of the TTP protein which influence where the protein binds along the ODC 3'UTR (Figure 5.4).

5.2.3 The ablation of TTP in mouse embryonic fibroblasts increases ODC mRNA stability without changing the ODC enzyme activity.

We next wanted to determine the effect knocking out TTP had on ODC mRNA stability. We used TTP knockout MEFs for these experiments (Taylor, Carballo et al. 1996). The ODC mRNA stability increased by 8-fold in TTP knockout MEFs when compared to wild-type MEFs (Figure 5.7). Interestingly, TTP wild-type MEFs displayed a 2-fold higher level of steady-state ODC mRNA when compared to TTP knockout MEFs (data not shown). No change in ODC enzyme activity was observed in these cells. Both cell lines exhibited basal levels of ODC enzyme activity (Figure 5.8). These data suggest that TTP decreases ODC mRNA stability, but that this change in ODC mRNA half-life does not alter the overall ODC enzyme activity in these cells. This could be explained by an increase in ODC translational efficiency in TTP wild-type MEFs and/or an increase in ODC protein degradation in the TTP knockout MEFs.
Figure 5.5. Cytoplasmic TTP binds to multiple binding sites along the ODC 3’UTR in A5 spindle carcinoma cells.

A5 cytoplasmic extracts were used in a biotin reaction in which 15 µl of biotin-labeled ODC overlapping fragments were incubated with 120 µg of cytoplasmic extract. The pulldown material was assessed for TTP binding by Western blot analysis. This experiment was done in duplicate with reproducible results. The full length (FL) ODC 3’UTR was used as a positive control.
Figure 5.6. Nuclear TTP in C5N cells binds to each fragment along the ODC 3'UTR.

Overlapping fragments, scanning the entire length of the ODC 3'UTR were used in this experiment. The fragments were biotin-labeled and incubated with 120 µg of C5N nuclear extract. TTP binding to each fragment was determined by Western blot analysis. This experiment was done in duplicate with reproducible results. Biotin-labeled 5' end and 3' end truncation studies of the ODC 3'UTR verified these results (data not shown). The full length ODC 3'UTR (FL) was used as a positive control.
Mouse embryonic fibroblasts (MEFs) were generated in which a functional TTP was ablated. TTP +/+ denotes MEFs containing functional TTP protein and TTP KO represents MEFs which have no functional TTP. Cells were treated with 10 µg/ml Actinomycin D. RNA was harvested at 0, 4, and 8 h after Actinomycin D treatment as described in the Materials and Methods chapter. qRT-PCR was used to measure the levels of ODC mRNA and Cyclophilin A mRNA. ODC mRNA levels were normalized to Cyclophilin A for each time point, and the ODC mRNA half-life was determined using exponential curve fit analysis. This experiment was done in duplicate with reproducible results (n=6).
Figure 5.8. ODC enzyme activity is not statistically different between TTP knockout and TTP wild-type mouse embryonic fibroblasts.

TTP wild-type (+/+) and TTP knockout (KO) cells were harvested at 48 h in ODC harvest buffer. An ODC enzyme activity assay was conducted as described in the Materials and Methods chapter. This experiment was conducted four times with reproducible results (n=17) (P=0.14).
Initial studies aimed to investigate the regulation of ODC by its UTRs in the two MEF cell lines were conducted. Measurement of luciferase activity was used to quantitate the influence of the ODC 3'UTR and 5'UTR in TTP knockout and wild-type MEFs. The constructs used were created by inserting cDNA encoding the ODC 3'UTR downstream of the luciferase gene in the pGL3 Control vector, or inserting the ODC 5'UTR upstream of the luciferase gene in the pGL3 Control vector (Figure 5.9). After a 48 h transient transfection, the Firefly luciferase activity was assayed and normalized to Renilla luciferase which had been co-transfected with the pGL3 plasmid. The luciferase activity measured in cells that had been transfected with the pGL3 Control vector which lacked both the ODC 3'UTR and ODC 5'UTR was set to 100%. Insertion of the ODC 3'UTR into the pGL3 Control vector resulted in a 75% reduction in luciferase activity in TTP wild-type cells and a 50% reduction in luciferase activity in TTP knockout cells, suggesting the presence of a negative regulatory element in the 3'UTR that is not dependent on TTP expression (Figure 5.9). The presence of the ODC 5'UTR resulted in a dramatic decrease in luciferase activity in both TTP knockout and TTP wild-type cells. TTP knockout cells displayed an 85% reduction in luciferase activity when compared to the pGL3 Control, and TTP wild-type cells showed a 95% reduction in luciferase activity when compared to the pGL3 Control (Figure 5.9). When comparing the influence of the ODC 5'UTR on luciferase activity in TTP knockout and TTP wild-type cells, a statistically significant change in luciferase activity was revealed, suggesting that the ODC 5'UTR contains a negative regulatory element, and that the negative regulation of the ODC 5'UTR on luciferase expression is TTP-dependent.

These data are in agreement with previous results shown by Lee et al. in which the 3'UTR of VEGF was fused to the luciferase gene in the pGL3 plasmid. When TTP was overexpressed in Colo320 cells the luciferase activity was markedly reduced in
Figure 5.9. The inclusion of either the ODC 5’UTR or ODC 3’UTR in the pGL3 Control vector causes a decrease in luciferase activity in mouse embryonic fibroblasts.

A. Schematic of the two luciferase constructs used. The ODC 5’UTR was placed upstream of the luciferase reporter gene and the ODC 3’UTR was placed downstream of the luciferase reporter gene. B. TTP knockout (KO) and TTP wild-type (+/+ ) mouse embryonic fibroblasts (MEFs) were plated at a density of 175,000 and 100,000 cells per 6 cm plate respectively. Cells were transiently transfected with either pGL3 Control, pODC3’UTRLuc, or pODC5’UTRLuc as described in the Materials and Methods chapter. Forty-eight h post-transfection, cells were assayed for luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity for each sample. The luciferase activity for cells transfected with pGL3 was set to 100%. Cells transfected with pODC3’UTRLuc and pODC5’UTRLuc are shown as a percent of the pGL3 activity. Values are the means ± S.E. This experiment was done in duplicate with reproducible results (n=7-10). **P<0.005.
comparison to the control pGL3 plasmid suggesting that TTP is a negative regulator of UTR mediated luciferase activity (Lee, Son et al. 2009).

5.2.4 The absence of TTP does not affect the localization or level of the HuR protein in mouse embryonic fibroblasts.

Since TTP and HuR can compete for binding to the same transcripts, we investigated the effect of TTP knockout on both HuR localization and the overall level of the HuR protein (Ming, Stoecklin et al. 2001; Al-Ahmadi, Al-Ghamdi et al. 2009). The absence of TTP did not affect the localization of the HuR protein, as both TTP knockout MEFs and TTP wild-type MEFs exhibited the same pattern of HuR expression. HuR was shown to be diffused in these cells, displaying both nuclear and cytoplasmic localization (Figure 5.10). Moreover, we measured the level of HuR protein in both cell lines because previous experiments showed that RBPs are capable of binding to and influencing the stability and translational efficiency of other RBPs (Al-Ahmadi, Al-Ghamdi et al. 2009). Based on these observations, we hypothesized that the HuR protein may be more abundant in TTP knockout cells because TTP is a destabilizing protein. However, we show that the level of HuR protein is constant in both TTP knockout and TTP wild-type MEFs, suggesting that the steady-state protein level of HuR is not impacted by TTP.

5.3 Discussion

The data described here support previous findings from our lab that the destabilizing RBP TTP binds to the ODC mRNA message. TTP, a CCCH-zinc finger RBP, has been shown to bind to and decrease the mRNA stability of numerous transcripts (Lai, Carballo et al. 1999; Carballo, Lai et al. 2000; Lai, Carballo et al. 2000; Stoecklin, Ming et al. 2000). Moreover, a reduction of the TTP protein, or the hyperphosphorylation of the TTP protein, has been correlated with a transformed
Figure 5.10. The absence of TTP does not affect the localization of HuR nor the level of HuR protein in mouse embryonic fibroblasts.

A, HuR localization does not change in the presence or absence of TTP. TTP wild-type (+/+) and TTP knockout (KO) cells were seeded onto glass coverslips that had been pre-coated with poly-L-lysine at a density of 20,000 and 40,000 respectively per 12 well plate. Forty-eight h later, cells were fixed according to the protocol described in the Materials and Methods chapter and processed for HuR immunofluorescence. Pictures were taken at 20X magnification on the Nikon Eclipse E800. This experiment was done in duplicate with reproducible results. B, TTP does not alter the endogenous protein levels of HuR. TTP +/+ and TTP KO cells were plated at a density of 90,000 and 67,000 cells per 6 cm plate respectively. Forty-eight h after plating, cells were harvested in 1X RIPA buffer. The level of HuR protein was determined by Western blot analysis. GAPDH was used as a loading control. A representative blot is shown. This experiment was done in duplicate with reproducible results.
phenotype in numerous cancers, including breast, ovarian, and malignant glioma (Suswam, Li et al. 2008; Brennan, Kuwano et al. 2009). Furthermore, in a study looking at TTP expression in colon cancer, it was found that TTP levels were dramatically decreased in adenocarcinoma samples when compared to non-tumorigenic controls. The level of TTP had an inverse relationship with a marker of neoplastic transformation, VEGF. VEGF protein levels were found to be elevated in adenocarcinoma samples and low in controls. TTP overexpression in Colo320 cells, a human colorectal cell line, correlated with a marked decrease in the mRNA stability of VEGF, a decrease in cell proliferation, and smaller tumors when these cells were injected into nude mice. These studies demonstrate that TTP negatively regulates tumorigenesis, and highlight TTP as a possible target for chemoprevention and chemotherapy (Lee, Son et al. 2009). It is hypothesized that the hyperphosphorylation of TTP mediated by the p-38-MAPK pathway causes TTP to bind to the 14-3-3 chaperone protein thereby making TTP unable to bind to its target mRNAs [Reviewed in (Sandler and Stoecklin 2008)].

Our studies show that cytoplasmic TTP binds to the ODC 3'UTR, CR, and 5'UTR in A5 spindle carcinoma cells but is only able to bind to the ODC CR in non-transformed C5N cells (Figure 5.1) (Dean, Wait et al. 2001; Meng, King et al. 2005). Nuclear TTP from C5N cells binds indiscriminately to the 5'UTR, CR, and 3'UTR of ODC (Figures 5.2 and 5.6). One possible explanation for these data is that TTP binds to the ODC mRNA in the nucleus to sequester the mRNA so that it cannot enter the cytoplasm and become translated or degraded. Because the overall level of TTP is similar between C5N and A5 cells, we can rule out the possibility that TTP is decreased in spindle carcinoma cells in order to promote tumorigenesis (Figure 5.3). However, we noted in our whole-cell lysates that TTP consistently runs as a doublet in C5N cells whereas it is primarily a single, higher molecular weight band in A5 cells, suggesting that TTP may be post-translationally modified in A5 cells. Moreover, a comparison of the nuclear and
cytoplasmic TTP protein in C5N and A5 cells revealed a similar banding pattern in both the nuclear and cytoplasmic fractions in A5 cells. The C5N nuclear fraction displayed a different banding pattern for TTP than the cytoplasmic fraction, again suggesting post-translational modifications to TTP (Figure 5.4). As mentioned above, TTP hyperphosphorylation correlates with increased tumor grade (Suswam, Li et al. 2008). Thus, we hypothesize that the TTP banding pattern we see in C5N and A5 cells is a result of phosphorylation. However, more studies investigating the over 20 putative phosphorylation sites on TTP need to be conducted in order to support this hypothesis of post-translational regulation of TTP in these cells (Cao, Deterding et al. 2007). Future studies include mass spectrometry to determine the phosphorylated residues in the C5N and A5 TTP proteins according to the protocol used by Chrestensen et al. (Chrestensen, Schroeder et al. 2004).

We show, irregardless of post-translational modifications, that the cytoplasmic A5 TTP binds to multiple binding sites along the ODC 3'UTR. The nuclear C5N TTP binds to all regions of the ODC 3'UTR (Figures 5.5 and 5.6). The ODC mRNA in A5 cells is stable, thus we would not expect a destabilizing protein like TTP to bind to the ODC 3'UTR strongly or at multiple binding sites. One explanation is that both HuR, or another stabilizing RBP, and TTP bind to the ODC 3'UTR in A5 cells. RBPs have been shown to bind to the same mRNA, and compete for binding sites (Blaxall, Dwyer-Nield et al. 2000; Liao, Hu et al. 2007; Al-Ahmadi, Al-Ghamdi et al. 2009). Lal et al. showed that HuR and the destabilizing RBP AUF1 bound to the 3'UTRs of p21 and Cyclin D1 at different, non-overlapping regions suggesting that RBPs can bind to the same mRNA simultaneously (Lal, Mazan-Mamczarz et al. 2004). A similar mechanism could be occurring in the A5 spindle carcinoma cells as HuR and TTP can bind to different regions of the ODC 3'UTR (Compare Figures 4.4 and 5.5). To determine whether the HuR and TTP proteins compete for similar binding sites, (F2), we could assess the binding of TTP to the ODC
3'UTR fragments in A5 cells that had been treated with siHuR. We have shown that siHuR decreases the ODC mRNA stability in A5 cells; however, we have not elucidated the mechanism behind this. One explanation is that a decrease in cellular HuR allows TTP to bind to regions of the ODC 3'UTR that are usually occupied by the HuR protein. Similarly, we can use siTTP to knockdown TTP in A5 cells and determine where HuR binds on the ODC 3'UTR. These experiments would reveal whether cytoplasmic TTP and HuR, two proteins associated with the ODC 3'UTR, compete for similar binding sites on the ODC 3'UTR or bind to different regions of the same transcript. Others have found that RBPs compete for labile mRNAs, thus it would not be surprising if HuR and TTP competitively bind to the ODC 3'UTR in A5 spindle carcinoma cells and C5N keratinocytes (Ming, Stoecklin et al. 2001; Lal, Mazan-Mamczarz et al. 2004; Liao, Hu et al. 2007).

To determine whether TTP phosphorylation aids in the binding of the protein to the ODC message, we could create phosphomimetic TTP mutants for all putative phosphorylation sites. TTP overexpressing plasmids would be transfected into TTP knockout MEFs and the biotin-labeled fragment mapping experiments would be utilized to determine whether phosphorylation of TTP changes the binding affinity or location of TTP binding to the ODC 3'UTR.

We demonstrate a functional correlation between TTP and ODC mRNA stability using TTP knockout MEFs. We show that the ablation of functional TTP results in a dramatic elevation in ODC mRNA stability (Figure 5.7). Interestingly, this increase in ODC mRNA stability does not result in an increase in ODC enzyme activity, as both TTP wild-type and TTP knockout cells display similar levels of ODC enzyme activity (Figure 5.8). These results suggest that ODC mRNA is translated more efficiently in TTP wild-type MEFs and/or that ODC protein degradation is increased in TTP knockout MEFs. Polysome analysis and ODC protein degradation studies using Cycloheximide could be
used in order to determine whether either of these mechanisms account for the observed ODC enzyme activity in these cells. Moreover, experiments using siTTP could be conducted in C5N and A5 cells in order to test the influence of TTP on ODC mRNA stability and ODC enzyme activity in a different cell model.

An initial study looking at the influence of the ODC 3’ UTR and 5’ UTR on luciferase activity revealed that both the 3’ UTR of ODC and the 5’ UTR of ODC contain negative regulatory elements, since the insertion of either region into the pGL3 Control plasmid dramatically reduced the level of luciferase activity in both TTP wild-type and knockout MEFs (Figure 5.9). However, the presence of TTP further reduced the 5’ UTR mediated luciferase activity, suggesting that TTP is a trans-negative regulatory element of the ODC 5’ UTR. To definitively show an affect of TTP on ODC protein synthesis, luciferase mRNA stability would need to be assessed in these cells. Moreover, polysome analysis would determine the effect of TTP on overall ODC translational efficiency.

Finally, immunofluorescence studies show that the cellular distribution of HuR is similar between TTP knockout and TTP wild-type MEFs, suggesting that TTP does not influence HuR cellular localization (Figure 5.10). Moreover, the HuR protein level is unchanged in TTP knockout and TTP wild-type MEFs, suggesting that TTP does not negatively regulate HuR in these cells (Al-Ahmadi, Al-Ghamdi et al. 2009).

We demonstrate that TTP, a destabilizing RBP, binds to the ODC transcript in A5 and C5N cells. Cytoplasmic TTP is able to bind to the ODC 3’ UTR, CR, and 5’ UTR in A5 spindle carcinoma cells and only binds to the ODC CR in C5N keratinocytes. This may be due to the phosphorylation of TTP because we know TTP is regulated by phosphorylation (Cao, Deterding et al. 2007; Sandler and Stoecklin 2008; Suswam, Li et al. 2008). Moreover, our results suggest that TTP is post-translationally modified in the cytoplasm of A5 cells because we see the presence of a higher molecular weight TTP
band in A5 cytoplasmic fractions when compared to C5N cytoplasmic fractions (Figure 5.4). We show in A5 cells that TTP binds to the ODC 3’UTR at regions that demonstrate HuR binding (F2), and also binds to sites that do not exhibit HuR binding (F5). These results support the hypothesis that HuR and TTP can both simultaneously and/or competitively bind to the ODC 3’UTR. Experiments to elucidate the relationship between HuR, TTP, and ODC are ongoing. Although the data described here raise many questions, they highlight the complex interplay of TTP, HuR, and ODC mRNA stability in the context of NMSC progression. Future studies will continue to investigate TTP, HuR, and ODC as valid targets for NMSC chemoprevention and chemotherapy.
CHAPTER 6
The ODC 3’UTR and 5’UTR contain \textit{cis}-regulatory elements

6.1 Introduction

Post-transcriptional regulation encompasses both mRNA stability and mRNA translation efficiency. We have shown that the cytoplasmic accumulation of HuR in A5 spindle carcinoma cells allows the HuR protein to bind to the ODC 3’UTR and thereby regulate the stability of the ODC mRNA (Chapter 4). In addition to regulating mRNA stability, HuR has been shown to influence the translational efficiency of its bound mRNAs. For example, HuR presence has been shown to increase the translational efficiency of c-Myc, MKP-1, and p53 (Kuwano, Kim et al. 2008; Liu, Rao et al. 2009; Tong and Pelling 2009). Thus, we started to investigate the influence of the ODC 3’UTR and ODC 5’UTR on luciferase activity in C5N and A5 cells as preliminary studies to look at ODC translational efficiency.

Studies investigating ODC regulation have shown that both the ODC 5’UTR and ODC 3’UTR control ODC mRNA translation. As previously mentioned, the ODC 5’UTR is inhibitory for ODC translation due to its length and extensive secondary structures (Manzella and Blackshear 1990; Van Steeg, Van Oostrom et al. 1991; Shantz and Pegg 1999). Lorenzini and Scheffler used constructs in which either the ODC 5’UTR, the 3’UTR, or both were inserted into the pRSV/L plasmid containing the Firefly luciferase reporter gene (Lorenzini and Scheffler 1997). ODC deficient Chinese hamster ovary (CHO) cells were transfected with these vectors, and the luciferase activity was measured in order to evaluate the influence of the ODC UTRs on translation. They showed that the ODC 5’UTR was inhibitory, and that the addition of the 3’UTR partially released this inhibition. The ODC 3’UTR also displayed a reduction in luciferase activity when compared to the control, but this inhibition was markedly less than the one
observed from the 5'UTR. These studies show that the 3'UTR and 5'UTR of ODC modulate ODC regulation (Lorenzini and Scheffler 1997). Our goal is to further these findings by investigating the influence of the ODC UTRs on luciferase activity in cells that contain endogenous ODC.

The data described here begin to study the influence of the ODC 5'UTR and ODC 3'UTR on luciferase activity in keratinocyte-derived cells. Insertion of the entire ODC 3'UTR and ODC 5'UTR into the pGL3 Control plasmid resulted in a decrease in luciferase activity in both the C5N keratinocytes and A5 spindle carcinoma cells. Deletion analysis identified the region between bases 1975 and 2135 in the ODC 3'UTR as inhibitory. Moreover, mutation of the AUUUA ARE in the ODC 3'UTR significantly increased luciferase activity, suggesting that the AUUUA sequence is a negative regulatory element on the ODC 3'UTR.

6.2 Results
6.2.1 The ODC 5'UTR and 3'UTR decrease the expression of the luciferase reporter gene.

To determine the effect of the ODC 3'UTR and 5'UTR on luciferase activity, reporter constructs were created by inserting the rat ODC 3'UTR or ODC 5'UTR cDNA into the pGL3 Control vector (constructs are denoted as pODC3'UTRLuc and pODC5'UTRLuc respectively) (Figure 6.1). The orientation of the ODC 5'UTR or ODC 3'UTR was verified by sequencing. Each plasmid contained the Firefly luciferase coding region under the control of the SV40 promoter.

To study the influence of the ODC UTRs on luciferase activity, we transfected both C5N cells and A5 cells with either the parental pGL3 Control vector, pODC3'UTRLuc, or pODC5'UTRLuc. A Renilla luciferase reporter vector was co-
Figure 6.1. Schematic of the reporter constructs used to determine the influence of the ODC 3'UTR and ODC 5'UTR on luciferase activity.

cDNA of the rat ODC 5'UTR was placed upstream of the luciferase reporter gene in the pGL3 Control vector (pODC5'UTRLuc). The rat ODC 3'UTR cDNA was placed downstream of the luciferase reporter gene in the pGL3 Control vector (pODC3'UTRLuc). As a control, the pGL3 Control vector was used. All constructs contained the Firefly luciferase reporter gene under the control of the SV40 promoter and enhancer elements, and contained the SV40 late poly(A) tail signal.
transfected into the cells to control for transfection efficiency. Firefly luciferase activity was measured and normalized to Renilla luciferase. The Firefly/Renilla ratio in cells transfected with the parental pGL3 Control vector was designated as 100%. We hypothesized that both the 3’UTR and 5’UTR would greatly reduce the luciferase activity in both cell lines. Based on previous data, we expected the ODC 5’UTR to be more inhibitory than the ODC 3’UTR (Lorenzini and Scheffler 1997). The insertion of the complete ODC 3’UTR resulted in an approximate 65% decrease in luciferase activity in both C5N and A5 cells (Figure 6.2). Insertion of the complete 5’UTR of ODC resulted in a more dramatic decline in luciferase activity in C5N cells. C5N cells showed a reduction in luciferase activity of approximately 80%. This is similar to previous results in CHO cells (Lorenzini and Scheffler 1997). However, A5 cells transfected with the ODC 5’UTR showed only a 20% reduction in luciferase activity when compared to the pGL3 Control vector (Figure 6.2). These data suggest the presence of cis-acting negative regulatory elements within the ODC 3’UTR and 5’UTR, because the reporter constructs contained identical promoter and enhancing elements. Moreover, these data suggest that trans-acting factors for the ODC 5’UTR are present as enhancers in A5 cell lysates or as inhibitors in C5N cell lysates because we see a markedly different effect of the 5’UTR on luciferase activity between these two cell lines, and because we know that the 5’UTR sequence is similar in the two cell lines (data not shown). This is in keeping with the higher ODC protein levels observed in A5 cells and would be consistent with an increase in ODC protein synthesis in these cells (Refer to Chapter 3).

6.2.2 The ODC 3’UTR contains a negative regulatory element between bases 1975 and 2135.

We decided to focus on the influence of the ODC 3’UTR on luciferase activity because research previously conducted concentrated on the role of the ODC 5’UTR,
Figure 6.2. Insertion of the ODC UTRs in the pGL3 Control vector causes a decrease in luciferase activity.

C5N keratinocytes and A5 spindle carcinoma cells were transfected with either the pGL3 Control vector, pODC3'UTRLuc, or pODC5'UTRLuc according to the protocol described in the Materials and Methods chapter. A plasmid containing the Renilla luciferase gene was co-transfected into these cells and used as a transfection efficiency control. Luciferase activity was measured 48 h after transfection. Firefly luciferase activity was normalized to Renilla luciferase for each sample. The luciferase activity of the pGL3 Control was set to 100% and the samples from cells transfected with pODC3'UTRLuc and pODC5'UTRLuc are shown as a percentage of the pGL3 Control luciferase activity. Values are means ± S.E. for 3 separate plates. The experiment was done in duplicate with reproducible results (n=6). **P<0.005.
and because we have shown that the ODC 3'UTR is important for ODC regulation through both cis and trans-acting factors (Refer to Chapter 4). To fully understand the impact of the ODC 3'UTR on luciferase activity, we measured the luciferase activity in C5N and A5 cells that had been transfected with either the full length ODC 3'UTR or two distal end truncation constructs (Figure 6.3). ARE02 (bases 1797-1975) and ARE03 (bases 1797-2135) both contain the classical AUUUA ARE (Refer to Figure 4.1).

Luciferase activity measured in cells transfected with the pGL3 Control vector lacking the 3'UTR of ODC was again set to 100%. The full length ODC 3'UTR exhibited a 70-80% decrease in luciferase activity. ARE02 displayed a 50% decrease in luciferase activity. However, luciferase activity was undetectable in cells transfected with ARE03. This trend was the similar between C5N and A5 cells (Figure 6.3). These data suggest that a negative cis-regulatory element resides between bases 1975 and 2135 on the ODC 3'UTR. Moreover, the data suggest that the most distal region of the ODC 3'UTR (bases 2135-2547) contains positive regulatory elements because we see a rescue in luciferase activity when we compare the full length 3'UTR to ARE03. This trend was also seen in rat intestinal epithelial (RIE-1) cells (Origanti and Shantz, unpublished data).

6.2.3 Mutation of the AUUUA classical ARE dramatically increases luciferase activity.

Next we investigated the effect of mutating the AUUUA ARE site on the ODC 3'UTR. These experiments were conducted to verify that the AUUUA site was a cis-acting regulatory element on the ODC 3'UTR. Using site-directed mutagenesis we mutated the AUUUA in the ARE02 vector to GGGUA (Figure 6.4). C5N cells were transfected with the pGL3 vector containing the full length ODC 3'UTR, ARE02 wild-type, which contained the AUUUA sequence, or ARE02 Mutant, which contained the GGGUA sequence. The luciferase activity of the pGL3 Control without the ODC 3'UTR was set to 100%. The full length ODC 3'UTR exhibited an 85% reduction in luciferase
Figure 6.3. The ODC 3’UTR contains a negative regulatory element between bases 1975 and 2135.

A. Schematic of the full length ODC 3’UTR and truncation constructs used in the luciferase experiments. Red dots denote the location of the AUUUA classical ARE sequence. B. Luciferase activity is decreased in plasmids that contain the full or truncated constructs of the ODC 3’UTR. The full length ODC 3’UTR (full 3’UTR) as well as two distal end truncations of the ODC 3’UTR were inserted into the pGL3 Control vector in order to show the influence of the 3’UTR of ODC on luciferase activity. This experiment was conducted in both C5N keratinocytes and A5 spindle carcinoma cells according to the protocol described in the Materials and Methods chapter. Each sample was normalized to Renilla. The pGL3 Control luciferase activity was set to 100%, and the full ODC 3’UTR, ARE02, and ARE03 data are represented as a percentage of the pGL3 Control luciferase activity. Values are means ± S.E. (n=6). This experiment was done in duplicate with reproducible results. Statistics were performed to compare the luciferase activity between Full 3’UTR, ARE02, and ARE03 in both cell lines. P<0.05 for all comparisons.
Figure 6.4. Mutating the AUUUA ARE to GGGUA increases the luciferase activity in C5N keratinocytes.

A, Schematic of the ARE02 wild-type (WT) and ARE02 Mutant constructs. The ARE02 WT construct is shown in Figure 6.3. Site-directed mutagenesis was used to change the AUUUA classical ARE sequence to GGGUA in the ARE02 vector. Sequencing confirmed the 3 base mutation. B, C5N cells were transfected with either the pGL3 Control vector, pODC3'UTRLuc (Full ODC 3'UTR), the ARE02 WT plasmid, or the ARE02 Mutant plasmid according to the protocols described in the Materials and Methods chapter. Cells were co-transfected with a plasmid containing the Renilla luciferase gene in order to measure transfection efficiency. The Firefly luciferase activity for each sample was normalized to Renilla luciferase. The luciferase activity of the pGL3 Control vector was set to 100%, and the luciferase activity of the Full ODC 3'UTR, ARE02 WT, and ARE02 Mutant constructs are shown as a percent of the pGL3 Control activity. Values are means ± S.E. for 3 independent plates. This experiment was done in triplicate with reproducible results (n=9). **P<0.005.
activity, while the ARE02 wild-type construct displayed a 45% reduction in luciferase activity. Interestingly, the ARE02 Mutant construct showed over 100% luciferase activity, suggesting that the AUUUA sequence is an inhibitory cis-acting regulatory element in the ODC 3'UTR.

6.3 Discussion

We have shown that the ODC UTRs contain negative regulatory elements. In agreement with previous studies conducted on the ODC UTRs, we show that the insertion of the 5'UTR of ODC and 3'UTR of ODC into the pGL3 Control vector causes a reduction in the luciferase activity (Figure 6.2) (Manzella and Blackshear 1990; Lorenzini and Scheffler 1997). It has been suggested that the insertion of the ODC 3'UTR in conjunction with the 5'UTR can relieve some of the repression exhibited by the 5'UTR (Greens and Scheffler 1990); this hypothesis is currently being investigated in our cell model.

We observed that insertion of the full length ODC 3'UTR dramatically reduces the luciferase activity. Moreover, a distal end truncation of the ODC 3'UTR, ARE02 (1797-1975), relieved some of the repression demonstrated by the full length 3'UTR. The ARE03 construct (1797-2135) completely ablated the luciferase activity. The addition of the most distal end of the ODC 3'UTR (2135-2547) relieves some of the repression measured in ARE03. This is observed when we compare the luciferase activities between ARE03 and the full length ODC 3'UTR (Figure 6.3). These data can be interpreted two ways. There are multiple positive and negative regulatory elements along the ODC 3'UTR which work in concert to regulate the ODC mRNA transcript, and/or the secondary structure of the ODC 3'UTR is altered in the truncation constructs which enhances or inhibits the binding of trans-acting factors.
Mutation of the ARE sequence AUUUA to GGGUA on the ODC 3’UTR resulted in a significant induction of luciferase activity. In fact, the luciferase activity observed in C5N cells that had been transfected with ARE02 Mutant was higher than the luciferase activity displayed in C5N cells that had been transfected with the pGL3 Control vector (Figure 6.4). These data are in agreement with studies conducted by Cok and Morrison. They found using the mouse COX-2 3’UTR that the removal of the first 60 nucleotides, which contained 7 out of 12 AUUUA sequences, caused an increase in luciferase activity. From these results they determined that the AUUUA consensus sequence was inhibitory (Cok and Morrison 2001). Our results recapitulate their findings and suggest that the AUUUA sequence is an inhibitory element on the ODC 3’UTR.

In these experiments, we began investigating the effects of the ODC 3’UTR and 5’UTR on luciferase activity. However, several experiments should be conducted in order to better define how the 3’UTR and 5’UTR affect ODC protein synthesis. We show that the pODC3’UTRLuc plasmid, in which the full length ODC 3’UTR is inserted downstream of the Firefly luciferase reporter gene, displays lower luciferase activity than the pGL3 Control vector when transfected into C5N and A5 cells. The changes in luciferase activity could be due to either alterations in mRNA stability or changes in the rate of translation. Based on our previous results we know endogenous ODC mRNA is stabilized in transformed A5 cells when compared to non-transformed C5N cells. In order to distinguish the effect of the 3’UTR on mRNA stability and translational efficiency, A5 and C5N cells would be transfected with the pODC3’UTRLuc plasmid and luciferase mRNA levels would be measured by qRT-PCR and normalized to Cyclophilin A at various time points after treatment with Actinomycin D. To test translation efficiency, \textit{in vitro} translation of luciferase controlled by the 3’UTR would be tested using a cell-free system (i.e. rabbit reticulocytes, wheat germ, or E.coli). We know from our current data that the luciferase activity in C5N and A5 cells is lower when cells are
transfected with pODC3’UTRLuc. These experiments will give us more information as to the mechanism responsible for this difference. To correlate the trans-acting factor HuR with the 3’UTR cis-regulatory elements we can also conduct the above experiments in the absence or presence of HuR knockdown. The results from these experiments would tell us how HuR affects the 3’UTR mediated mRNA stability and translational efficiency of the ODC mRNA transcript.

In conjunction with studying the effect of the 3’UTR on ODC regulation, studying regulation by the 5’UTR would be interesting. A report by Meng et al. showed that HuR was able to bind to the 5’UTR of the type I insulin-like growth factor receptor (IGF-IR). Like ODC, the IGF-IR 5’UTR is very long (approximately 1038 nucleotides in length). HuR binding to the 5’UTR of IGF-IR repressed translation initiation and also repressed IRES-mediated translation (Meng, King et al. 2005). Interestingly, previous results from our lab have shown that the ODC 5’UTR may contain an IRES (Origanti and Shantz 2007). We have shown in C5N cells that HuR binds to the 5’UTR of ODC (Figure 4.9). Furthermore, Manzella and Blackshear have shown that the rat ODC 5’UTR binds to an unknown RBP. The cytoplasmic fractions from numerous animal species including human, mouse, cow, chicken, and frog showed binding of this protein to the ODC 5’UTR was similar amongst species, and the species tested contained a conserved CCAUCUC sequence in the ODC 5’UTR (Manzella and Blackshear 1992). These data suggest that the 5’UTR can influence both the mRNA stability and translational efficiency of labile mRNAs. Studies in which the pODC5’UTRLuc is transfected into the A5 and C5N cells could be conducted in a manner similar to the ones described above for studying the 3’UTR influence on ODC protein synthesis. Moreover, RBPs can bind to both the 5’UTR and 3’UTR of the mRNA transcript and regulate mRNA stability and translation. Luciferase activity studies could be done in the
presence or absence of HuR knockdown in order to determine whether HuR mediates ODC regulation through the 5'UTR.

The ODC translation efficiency in non-transformed C5N and transformed A5 cells should be measured to determine how ODC is post-transcriptionally regulated during skin tumor development. These experiments could be accomplished two ways. We could create a vector in which both the 5'UTR and 3'UTR surround the luciferase reporter gene and then determine the luciferase activity of this construct in both C5N and A5 cells. The steady-state luciferase mRNA level and the luciferase mRNA stability would be measured in order to demonstrate whether the changes observed in luciferase activity were due to translation or mRNA stability. An alternate experiment would be polysome analysis of ODC mRNA in both the C5N and A5 cells. This would directly tell us how efficiently the mRNA was being translated in each cell line. Furthermore, using RNAi we could assess the ODC translation efficiency in A5 cells in the presence or absence of HuR knockdown. We know that HuR binding directly impacts ODC mRNA stability in A5 spindle carcinoma cells. Thus, it would be interesting to determine if HuR also directly influences ODC mRNA translational efficiency. The data from these studies would provide not only a better understanding of ODC regulation during skin tumor development but would also provide a more comprehensive mechanism of how HuR affects ODC expression.

The studies described here demonstrate that both the ODC 3'UTR and 5'UTR influence luciferase activity, thereby adding to the complexity of ODC regulation. We show that both positive and negative regulatory elements are contained within the UTRs of ODC. We are currently investigating the mechanisms of ODC 3'UTR and 5'UTR mediated regulation, and how HuR is involved in these processes.
7.1 General summary and conclusions

The main goal of this work was to utilize a mouse keratinocyte model derived during the development of skin carcinomas in order to better understand ODC regulation in skin tumor development. We found that ODC enzyme activity was elevated in A5 spindle carcinoma cells when compared to non-transformed C5N keratinocytes, despite C5N cells exhibiting a slightly higher rate of ODC transcription (Figures 3.2 and 3.4). We reasoned that the upregulation of ODC enzyme activity in A5 spindle carcinoma cells could be explained by an increase in the mRNA stability of the ODC message in A5 cells, increased translational efficiency of ODC mRNA in A5 cells, post-translational modifications to ODC, and/or a decrease in the degradation of ODC protein in A5 cells.

We started investigating the mechanism of increased ODC enzyme activity by measuring the ODC mRNA stability in non-transformed C5N keratinocytes and transformed A5 spindle carcinoma cells. A5 cells displayed a marked increase in ODC mRNA stability when compared to the C5N cells (Figure 3.5). These data are novel as ODC has not been shown to be regulated at the level of mRNA stability. Based on this result, we became interested in the post-transcriptional regulation of ODC.

During post-transcriptional regulation, a fully processed mRNA shuttles into the cytoplasm where it can undergo various fates: it can be degraded, it can be translated into protein, or an RNA binding protein (RBP) can bind to the 3'UTR of the message and either delay degradation by inhibiting the decay machinery from binding to the mRNA transcript, influence translation efficiency, or shuttle the mRNA to a P-body for storage. RBPs classically bind to adenosine- and uracil-rich elements (AREs) and upon examination of the ODC 3'UTR a classic AUUUA ARE sequence was located (Figure
Furthermore, it has been shown that polyamine depletion leads to the cytoplasmic accumulation of the RBP HuR (Zou, Mazan-Mamczarz et al. 2006; Xiao, Rao et al. 2007; Zou, Liu et al. 2008; Liu, Rao et al. 2009). These findings led us to study HuR binding to the ODC 3′UTR in both the C5N keratinocytes and A5 spindle carcinoma cells. Both biotin-labeled synthetic ODC 3′UTR pulldown assays and endogenous mRNP assays revealed that cytoplasmic HuR was bound to the ODC 3′UTR in transformed A5 cells but was not bound to the ODC 3′UTR in C5N cells (Figures 4.2 and 4.3). Since the sequence for the ODC 3′UTR was found to be identical in both cell lines, and the whole-cell level of HuR was equal in C5N and A5 cells (Figures 4.2 and 4.7), we investigated the intracellular localization of HuR. HuR is a mobile protein, shuttling between the nucleus and cytoplasm in response to various stimuli (Kuwano, Kim et al. 2008; Zhang and Bowden 2008). Furthermore, increased cytoplasmic HuR correlates with increased tumor grade and poor disease prognosis in numerous types of cancer (Erkinheimo, Lassus et al. 2003; Heinonen, Bono et al. 2005; Koljonen, Bohling et al. 2008). Thus, we hypothesized that the discrepancy in binding status was due to the localization of HuR. Immunofluorescence revealed that HuR was primarily nuclear in C5N cells and was both nuclear and cytoplasmic in A5 spindle carcinoma cells, supporting this hypothesis (Figure 4.8).

To better characterize the binding site of HuR on the ODC 3′UTR we conducted numerous mapping experiments using A5 and C5N extracts. Both sets of mapping data revealed that HuR binds to the ODC 3′UTR between bases 1851 and 2151 (Figures 4.4, 4.5, 4.6, 4.10, 4.11). These data suggest the presence of multiple HuR binding sites on the ODC 3′UTR.

To definitively link HuR localization to function, we used RNAi to knockdown HuR in both C5N and A5 cells (Figures 4.12 and 4.14). Knockdown of HuR in C5N cells did not change ODC mRNA stability; however, in A5 cells we saw a drastic decrease in both
ODC enzyme activity and ODC mRNA stability in cells treated with siHuR (Figure 4.13). These data indicate that the cytoplasmic accumulation of HuR in A5 cells increases the ODC mRNA half-life by binding to the ODC transcript.

HuR localization has been shown to be influenced by upstream cascades such as PKC and AMPK [Reviewed in (Doller, Pfeilschifter et al. 2008)]. Accordingly, we decided to determine whether these upstream kinases, as well as mTOR, which is downstream of AMPK, altered HuR localization in C5N and A5 cells. Previous data has shown that HuR localization was altered when AMPK was either activated or inhibited (Wang, Fan et al. 2002; Wang, Yang et al. 2004). The addition of Rapamycin was shown to decrease VEGF mRNA stability in two renal cancer cell lines (786-O and Cak-1 cells), suggesting that mTOR and RBPs may play a role in mediating the mRNA stability of labile mRNA transcripts (Basu, Datta et al. 2010). We know HuR is primarily regulated by its localization [Reviewed in (Brennan and Steitz 2001; Hinman and Lou 2008)], and so hypothesized that Rapamycin decreases mRNA stability by altering the localization of RBPs. Based on these two sets of data, we decided to investigate the role of mTORC1 in HuR localization. Rapamycin, an mTORC1 inhibitor, was used in A5 spindle carcinoma cells in an attempt to cause the movement of HuR into the nucleus. We showed that ODC enzyme activity was reduced in A5 cells in the presence of Rapamycin (Figure 4.17). However, Rapamycin treatment did not cause HuR localization to change in A5 cells, nor did it alter ODC mRNA stability (Figure 4.19).

Similarly, the PKC activator TPA did not cause the cytoplasmic accumulation of HuR in C5N cells, nor did the AMPK activator AICAR (Figures 4.20 and 4.21). Thus, the cellular events that induce the cytoplasmic localization of HuR in A5 spindle carcinoma cells remain elusive.

In addition to increasing mRNA stability, HuR has been shown to influence the translational efficiency of a number of its bound mRNA transcripts (Kuwano, Kim et al.
2008; Mazan-Mamczarz, Hagner et al. 2008). Thus, we decided to conduct preliminary studies to determine the influence of the ODC 3’UTR and 5’UTR on luciferase activity, as it has been previously suggested that the ODC 5’UTR and 3’UTR can regulate luciferase activity (Manzella and Blackshear 1990; Lorenzini and Scheffler 1997; Lovkvist Wallstrom, Takao et al. 2001). The full length 5’UTR of ODC was placed upstream of the Firefly luciferase coding region, while the full length ODC 3’UTR was placed downstream of the Firefly luciferase coding region (Figure 6.1). The luciferase activity measured in cells transfected with the full length ODC 3’UTR resulted in an approximately 65% reduction in luciferase activity when compared to cells transfected with the pGL3 Control vector. This trend was observed in both C5N and A5 cells, and suggests the presence of negative regulatory elements in the ODC full length 3’UTR that are not cell-type dependent (Figure 6.2). These data are similar to a previous study in which the full length COX-2 3’UTR was shown to have an inhibitory affect on luciferase expression (Cok and Morrison 2001). In contrast, luciferase activity was significantly higher in A5 cells transfected with the full length ODC 5’UTR than in C5N cells transfected with the full length ODC 5’UTR, suggesting the presence of a negative trans-acting factor in C5N cells or a trans-enhancing factor in A5 cells. Alternatively, the same trans-acting factor, such as RBPs, may bind differently to the 5’UTR in C5N versus A5 cells and thereby impact the effect of the ODC 5’UTR in protein synthesis.

When we more closely investigated the influence of the ODC 3’UTR on luciferase activity, we discovered that the region between bases 1975 and 2135 on the ODC 3’UTR contained negative regulatory elements. The luciferase activity drastically decreased when C5N and A5 cells were transfected with the ARE03 plasmid that contained bases 1797-2135 of the ODC 3’UTR when compared to the luciferase activity of cells transfected with ARE02 which contained bases 1797-1975 of the ODC 3’UTR (Figure 6.3). Moreover, we saw a partial relief of luciferase activity repression when the
full length ODC 3’UTR was transfected, suggesting that the region between bases 1975 and 2135 contains a negative regulatory element (Figure 6.3). We found that the AUUUA containing region of the ODC 3’UTR is inhibitory. Mutating the AUUUA to GGGUA significantly increased the luciferase activity in C5N cells (Figure 6.4). These data, although preliminary, suggest the presence of translationally inhibitory cis-acting elements on the ODC 3’UTR. One way of explaining these data is that mutating the AUUUA deletes the binding site for destabilizing RBPs, thereby increasing the ODC protein synthesis rate by stabilizing its mRNA. Furthermore, deletion of the ODC 3’UTR between bases 1975 and 2135 could render a similar effect by destroying a binding site(s) for destabilizing RBPs, thus promoting an increase in luciferase activity.

It has been shown that numerous RBPs can bind to the same 3’UTR at similar or different sites. For example, two RBPs AUF1 and TIAR were shown to competitively bind to the 3’UTR of c-Myc (Liao, Hu et al. 2007). Our lab discovered that the destabilizing protein TTP is able to bind to the ODC 3’UTR in wild-type and Ras transformed RIE-1 cells (Origanti and Shantz, unpublished data). We therefore wanted to determine whether TTP was able to bind to the ODC mRNA transcript in C5N keratinocytes and A5 spindle carcinoma cells. The coding region of ODC was shown to associate strongly with cytoplasmic TTP in C5N cells. Cytoplasmic TTP in A5 cells binds to the ODC 5’UTR, coding region, and 3’UTR (Figure 5.1). Mapping experiments using A5 cytoplasmic extracts revealed that TTP binds to multiple sites along the full length ODC 3’UTR (Figure 5.5). Interestingly, TTP showed binding to the most distal fragment of the ODC 3’UTR, in contrast to HuR, which does not bind to this region (Figures 4.4, 4.5, 4.6, and 5.5). The mapping results also revealed that both TTP and HuR bind to a similar location on the ODC 3’UTR in A5 cells (F2). Mapping studies using C5N nuclear extracts showed TTP strongly binding to the coding region and ODC 3’UTR (Figure 5.2). However, we show that TTP present in C5N nuclear extracts binds
to every region of the ODC 3’UTR (Figure 5.6). Thus, we hypothesize that TTP is post-
translationally modified differently in A5 cytoplasmic extracts and C5N nuclear extracts.
Further investigation is required to elucidate this mechanism.

We obtained mouse embryonic fibroblasts (MEFs) in which functional TTP was
deleted (a generous gift from Dr. Perry J. Blackshear, NIEHS) (Taylor, Carballo et al.
1996). These cells were used to determine the effect of TTP on ODC mRNA stability.
We hypothesized that knocking out a functional TTP protein would stabilize the ODC
mRNA transcript. Our results show an 8-fold increase in ODC mRNA stability in TTP
knockout cells when compared to TTP wild-type cells (Figure 5.7). However, the
increase in ODC mRNA stability does not correlate with an increase in ODC enzyme
activity, as both cell lines maintain low ODC enzyme activity levels (Figure 5.8).
Moreover, when we investigated luciferase activity in these cells using the pODC-
3’UTRLuc and pODC5’UTRLuc plasmids, we see results that are similar to those shown
in the C5N and A5 cell lines. Transfection of full length ODC 3’UTR resulted in an
approximately 70% decrease in luciferase activity in both TTP wild-type and TTP
knockout MEFs when compared to cells transfected with pGL3 Control, again
suggesting that the 3’UTR of ODC contains negative regulatory elements. Furthermore,
in cells transfected with the full length ODC 5’UTR we see an 85-95% decrease in
luciferase activity in the TTP knockout MEFs and TTP wild-type MEFs respectively,
suggesting that both the ODC 3’UTR and ODC 5’UTR contain negative regulatory
elements (Figure 5.9).

The data described here are novel as ODC has never before been shown to be
regulated by an RBP binding to its 3’UTR. We demonstrate a new mechanism of ODC
regulation by describing HuR binding to the ODC 3’UTR in a mouse model of non-
melanoma skin cancer. We show that ODC mRNA stability is enhanced in A5 spindle
carcinoma cells due to the cytoplasmic accumulation of HuR. This does not occur in
non-transformed cells, where HuR is predominately nuclear. Based on our data we
developed a model, shown as Figure 7.1, by which either UVB or the multi-stage
cellular carcinogenesis model causes HuR accumulation in the cytoplasm due to the
post-translational modification of HuR at a residue located in the HuR nucleocytoplasmic
shuttling (HNS) sequence or due to a modification to the protein(s) which
imports/exports HuR in and out of the nucleus. We hypothesize that HuR is post-
translationally modified and not mutated in A5 cells because HuR has never been shown
to be mutated in cancer (Lopez de Silanes, Lal et al. 2005). The upstream pathway
responsible for the HNS modification is currently unknown and needs to be elucidated.
The localization of HuR in the cytoplasm allows HuR to bind to numerous transcripts, as
shown in Figure 4.15, many of which contribute to tumorigenesis. One HuR target
transcript we were interested in further characterizing was ODC. Our data show that
cytoplasmic HuR binds to the ODC mRNA at its 3'UTR and causes an increase in ODC
mRNA stability. It is possible that HuR also increases the translational efficiency of the
ODC transcript. Experiments to definitively determine this are ongoing.

7.2 Future directions

Our studies provide interesting insight and open up numerous experiments for
further investigation of post-transcriptional regulation during NMSC. This section
reviews some of the directions that would further characterize this mechanism of ODC
regulation. The experiments described here could also be extended to include studying
the mechanism of post-transcriptional regulation for other transcripts that are important
in skin carcinogenesis.
Non-transformed keratinocytes from mice that have not undergone the multi-stage chemical carcinogenesis protocol or photocarcinogenesis exhibit short ODC mRNA half-lives, low ODC enzyme activity, and nuclear HuR. Transformed keratinocytes are from mice that have undergone either the multi-stage chemical carcinogenesis protocol or the photocarcinogenesis protocol. During tumor development, upstream pathways post-translationally modify HuR. The HuR protein shuttles out of the nucleus and into the cytoplasm where it can bind to numerous labile mRNA transcripts including ODC. HuR binds to the ODC mRNA transcript and causes an increase in ODC mRNA stability. Additionally, the binding of HuR to the ODC mRNA correlates with an increase in ODC enzyme activity.
7.2.1 Other possible mechanisms of post-transcriptional regulation in NMSC development.

As previously mentioned, the term post-transcriptional regulation refers to several mechanisms that regulate a labile mRNA. Post-transcriptional regulation encompasses mRNA processing, mRNA degradation, mRNA translation, and P-body storage. The studies described in this thesis have centered around ODC mRNA degradation in non-transformed and transformed cells in NMSC. Possible routes of future investigation are reviewed here.

7.2.1.1 MicroRNA

MicroRNAs (miRs) are considered major players involved in the post-transcriptional regulation of labile mRNAs. miRs are approximately 22 nucleotide-long, single-stranded, non-coding regulators of gene expression. These endogenous RNAs target cytoplasmic mRNAs of interest by imperfect base pairing and trigger endonuclease cleavage, translational repression, or accelerate mRNA decapping. Specifically in eukaryotes, miR-mRNA interactions involve contiguous Watson-Crick base pairing of the miR 5’ seed region (residues 2-8) to the mRNA, and a lack of complementarity in the central part of the miR (residues 10-12) [Reviewed in (Valencia-Sanchez, Liu et al. 2006; Pillai, Bhattacharyya et al. 2007; Fabian, Sonenberg et al. 2010)].

It is estimated that 700 or more miRs regulate 30% of human genes [Reviewed in (Garzon, Calin et al. 2009; Grady and Tewari 2010)]. Thus, it is highly probable that ODC mRNA is regulated by miRs. In fact, when using a miR binding site predicting database (microRNA.org) several sites on the ODC 3’UTR were highlighted as potential miR binding site hits. Although our studies have not focused on miR regulation of ODC, it is noteworthy that in addition to RBPs, miRs also bind to the 3’UTR of labile mRNAs and regulate decay and translation. Interestingly, it has been shown that HuR can
regulate miR-mediated repression. Bhattacharyya et al. demonstrated that in human hepatoma, Huh7 cells, cationic amino acid transporter (CAT-1) was regulated by miR-122, a liver specific miR (Bhattacharyya, Habermacher et al. 2006). They showed that miR-122 translationally repressed CAT-1, and noted that during cellular stress (oxidative stress or amino acid depletion) CAT-1 was relieved of its repression by miR-122. This was due to HuR shuttling to the cytoplasm during the stress response and binding to the CAT-1 mRNA. Knockdown of HuR prevented the stress stimulated derepression of CAT-1. The authors hypothesized that HuR interfered with the function of the miR either by binding to the miR-122 binding site or by changing the secondary structure of the mRNA upon binding, and thereby abating the miR-122 binding site. These data suggest that RBPs and miR either work together, or compete, in order to influence the regulation of target mRNAs, and that these two trans-regulatory elements often target the same mRNA.

MiRs are also involved in tumor development. Numerous miRs are aberrantly expressed in cancer (Volinia, Calin et al. 2006; Gaur, Jewell et al. 2007). For example, miR-21 and let-7a have been highlighted as two miRs that are deregulated in various types of cancer (Volinia, Calin et al. 2006; Garzon, Calin et al. 2009; Grady and Tewari 2010). Expression patterns of the MicroRNAome have shown that there are differences in the miR signatures of tumor and control samples. Moreover, there are variations in miR signatures between different tumor types (Volinia, Calin et al. 2006; Gaur, Jewell et al. 2007). The targets of miRs often include oncogenes and tumor suppressor genes thereby providing more evidence that miR regulation is important for understanding cancer biology, and further highlighting miRs as potential early biomarkers of cancer and targets for therapy [Reviewed in (Garzon, Calin et al. 2009; Grady and Tewari 2010)].

Currently, no published accounts of miR deregulation in NMSC are available. Thus, potential miRs involved in NMSC tumor development would be chosen based on
miRs known to be deregulated in either melanoma and/or other epithelial cancers. For example, it is known that miR-200 and miR-205 are highly expressed in normal skin (Yi and Fuchs 2010). Thus, these two miRs may be interesting to study in C5N and A5 cells. Moreover, using miR predicting databases we could study putative miRs associated with NMSC development based on a gene of interest. Specifically for ODC, we found that human miR-181a, miR-302, and miR-105 were predicted to bind to the ODC 3'UTR. Another approach for choosing miRs involved in NMSC is to conduct a miR array on C5N and A5 cells and determine which miRs are deregulated in A5 cells when compared to C5N cells.

To study potential miRs which bind to the ODC mRNA transcript, or other transcripts of interest which are deregulated in NMSC, we would use pre-miRs, which are processed into mature miRs, and antisense miRs for the miR family we were interested in studying. The subsequent work would mimic studies done by Abdelmohsen et al., who examined the influence of miR-519 on HuR regulation (Abdelmohsen, Srikantan et al. 2008). They found that in numerous cancer cell lines including HCT116 and RKO, both colon cell lines, that miR-519 expression was induced and that this induction of the miR caused a reduction in HuR levels. Moreover, knockdown of miR-519 caused an elevation in HuR levels, which was due to an increase in translational efficiency and not due to mRNA stability (Abdelmohsen, Srikantan et al. 2008). Thus, using similar methods, we would be able to determine the effects of any miR on mRNA stability and translation thereby showing another mechanism of regulation in NMSC development.

As described above there is much interplay between labile mRNA transcripts, RBPs, and miRs. Studies have been conducted that describe how RBPs interfere with the function of miRs (Bhattacharyya, Habermacher et al. 2006) and conversely, how miRs influence the expression of RBPs (Abdelmohsen, Srikantan et al. 2008). It is likely
that all of these mechanisms are occurring in NMSC development, thus it is imperative to continue to study the role of post-transcriptional regulation in the development of a tumorigenic phenotype in order to design chemotherapeutics with high efficacy.

7.2.1.2 P-bodies

P-body formation in both C5N and A5 cells is another potentially interesting line of investigation. P-bodies are aggregates of translationally repressed mRNAs that are usually bound by distinct RBPs. Labile mRNAs in P-bodies can either be degraded or can re-enter the cytoplasm for translation (Brengues, Teixeira et al. 2005). Global translation is often increased during tumorigenesis due to an increase in the expression of translation factors such as eIF4E and phosphorylated eIF4E as well as the downregulation of 4E-BP through its hyperphosphorylation [Reviewed in (Silvera, Formenti et al. 2010)]. In A5 cells we have shown an increase in phosphorylated 4E-BP (unpublished data) and therefore predict that A5 cells would contain fewer P-bodies than C5N cells because P-body number has been shown to decline during translation (Brengues, Teixeira et al. 2005; Parker and Sheth 2007; Franks and Lykke-Andersen 2008). This hypothesis would be tested by determining the number of P-bodies in both C5N and A5 cells using immunofluorescence for the P-body components Dcp1 and/or XRN1, and we would try to correlate P-body number with cellular transformation.

P-bodies are dynamic structures which form and dissociate rapidly in response to various cellular cues [Reviewed in (Parker and Sheth 2007; Franks and Lykke-Andersen 2008; Kulkarni, Ozgur et al. 2010)]. The mechanisms behind P-body assembly and disassembly remain an enigma. We propose studying the effects of various pharmacological agents on P-body assembly in A5 spindle carcinoma cells. Our ultimate goal would be to enrich P-bodies within A5 cells. We predict that the formation of P-bodies in A5 cells would shift these cells from highly proliferating cells to less tumorigenic cells by stimulating either the decay or sequestration of mRNA transcripts.
associated with tumorigenesis [Reviewed in (Franks and Lykke-Andersen 2008; Kulkarni, Ozgur et al. 2010)]. Pharmacological agents of interest would include Rapamycin, which blocks 4E-BP1 phosphorylation, and the MEK inhibitor PD98059 because MEK is a downstream target of Ras which is activated in the A5 cells. Moreover, RNAi could be used to determine whether mTOR or MEK are responsible for P-body assembly. These experiments would not only enhance our understanding of P-body assembly, but would contribute to the field of carcinogenesis by introducing another target for chemotherapies.

7.2.2 Determining upstream cascades which impact HuR localization.

In Chapter 4 we began to investigate several pathways which could alter the localization of HuR in C5N and A5 cell lines. Based on previous data, we looked at the effects of AMPK and PKC on HuR localization [Reviewed in (Doller, Pfeilschifter et al. 2008)]. Our findings suggested that neither pathway was responsible for HuR localization in non-transformed C5N cells or transformed A5 spindle carcinoma cells. In order to fully rule out these pathways we could use an RNAi approach because RNAi is more specific than most pharmacological agents. We would assess the localization of HuR in these and non-siRNA treated cells to determine whether PKC or AMPK was upstream of HuR. We would expect to see changes in the localization of HuR in cells that had been treated with siAMPK or siPKC when compared to non-treated cells if either pathway is a mediator of HuR localization.

We further explored whether the mTORC1 pathway was involved in HuR shuttling by treating A5 spindle carcinoma cells with Rapamycin. We hypothesized that treatment with Rapamycin would cause the nuclear accumulation of HuR. No change in HuR was observed in A5 cells treated with 100 nM of Rapamycin, suggesting that mTORC1 does not mediate the localization of the HuR protein. However, in order to definitively rule out mTORC1 as an upstream mediator of HuR localization, we could
measure HuR localization in the presence or absence of siRaptor, an mTORC1 specific component. Moreover, future complementary work could investigate the role of mTORC2 on HuR localization by utilizing Rictor knockout MEFs. siAKT was previously shown to decrease HuR levels in MKN28 cells while overexpressing AKT caused an increase in HuR levels in SNU216 cells. Moreover, the increase in cytoplasmic HuR following serum stimulation was attenuated by the presence of siAKT, suggesting that AKT plays a role in the shuttling of HuR (Kang, Ryu et al. 2008). As AKT is a phosphorylation target of mTORC2, it is possible that mTORC2 is an upstream kinase which influences HuR localization. The Rictor knockout MEFs as well as the wild-type MEFs could be used to assess HuR localization. If HuR localization was different between Rictor knockout and wild-type MEFs we could similarly knockout Rictor in the C5N and A5 cells and determine the HuR localization.

Finally, another potential regulator of HuR localization is the MEK pathway. MEK is downstream of activated Ras. A5 spindle carcinoma cells carry a mutated Ras at codon 61, whereas C5N cells contain wild-type Ras (Zoumpourlis, Solakidi et al. 2003). Thus, MEK is a good candidate for post-translationally modifying HuR. The MEK inhibitor, U0126, could be used in both cell lines and HuR localization could be determined at various time points. If we find that the MEK inhibitor causes changes in the localization of HuR we would use siRNA against MEK to further demonstrate its effect on HuR localization. The studies described here could be used to look at the upstream mediators of HuR localization not only in NMSC, but other epithelial cancers as RNAi approaches and immunofluorescence are optimized for most cell types. In addition to the pathways described above, other pathways implicated in tumorigenesis could be tested for their role in HuR localization.
7.2.3  *In vitro studies of ODC regulation in NMSC using P1 and B9 cells.*

We show in Appendix B, that there are two additional cell lines in our NMSC model which represent tumor promotion (C5N, P1, B9, A5). P1 cells were isolated from the papillomas of mice that had been treated with DMBA and TPA, while B9 cells were isolated from the squamous carcinomas of mice that had been treated with DMBA and TPA (Zoumpourlis, Solakidi et al. 2003). Although many preliminary studies have already been conducted in these cells, we could conduct biotin-labeled pulldowns and mRNP assays in order to determine whether HuR binds to the ODC 3’UTR in these cells. Moreover, we could use HuR knockdown to assess the effect HuR has on ODC enzyme activity, ODC mRNA stability, and ODC translation in both cell lines. The experiments conducted in these cells would provide us with a more comprehensive picture as to when and how HuR regulates ODC mRNA during tumorigenesis.

7.2.4  *In vivo studies of ODC regulation in NMSC.*

The work described here uses an *in vitro* model to study the regulation of ODC by the RBP HuR. In order to make these findings more applicable for chemoprevention and chemotherapeutic studies, HuR expression in mice which have developed NMSC should be studied. FVB mice would be used for these studies because this strain not only develops benign skin papillomas, but displays a high rate of progression from papilloma to carcinoma (Hennings, Glick et al. 1993). This would allow us to not only look at HuR and ODC in the context of tumor promotion, but would also allow us to assess HuR localization and ODC enzyme activity through tumor progression. Using the classical multi-stage DMBA/TPA protocol, we would measure the rate of tumor development, as well as the size of the tumors formed. A subset of mice that had developed papillomas would be sacrificed and their tumors would be excised for ODC enzyme activity assays and HuR immunohistochemistry. Other mice would continue
with TPA treatments to allow for the conversion of papilloma to carcinoma. Carcinomas would be harvested from mice for ODC enzyme activity assays and HuR immunohistochemistry. Skin of untreated control mice would be assayed for ODC enzyme activity and HuR immunohistochemistry in order to provide an ODC activity baseline and to determine whether the localization of HuR changes during tumor development. Moreover, the tumors from these mice could be used to study the regulation of other mRNAs that are associated with tumorigenesis, and that have been shown to bind to HuR. The overall goal of these studies would be to show that HuR localization does change in vivo during NMSC, and determine whether HuR localization correlates with tumor grade. We could also treat the mice topically with pharmacological agents which cause the regression of skin tumors and determine the localization of HuR in these cells.

In addition to using the multi-stage chemical carcinogenesis model described above, FVB mice could be treated with repeated doses of UVB in order to form tumors by photocarcinogenesis. This would provide a more physiologically relevant model of NMSC, since UV exposure is the likely carcinogen in human NMSC development. Similar to the multi-stage chemical carcinogenesis studies, mouse tumors would be harvested for ODC enzyme activity assays and HuR immunohistochemistry studies. We would expect that tumors would display higher ODC enzyme activity and an accumulation of cytoplasmic HuR when compared to the skin of sham-irradiated control mouse.

Animal studies could be expanded to include mice in which HuR is conditionally knocked out in the skin. Katsanou et al. created HuR$^{fl/fl}$ mice in which the second exon of HuR was flanked by the loxP sequence (Figure 7.2) (Katsanou, Milatos et al. 2009). Our mouse model would include the use of these mice and deleter Cre transgenic mice.
Figure 7.2. Schematic of the HuR locus with intron/exon boundaries and loxP sites.

HuR\(^{+}\) represents a partial schematic of the HuR gene which contains 6 exons and is located on mouse chromosome 8. HuR\(^{\text{fl}}\) represents a construct created in which the second exon of HuR is flanked by loxP sequences and a neomycin resistance gene. HuR\(^{-}\) is produced by standard targeting procedures in which the HuR\(^{\text{fl}}\) construct was placed into an embryonic stem cell. These mice had a fully functional HuR allele. HuR\(^{-}\) represents the Cre-recombined locus that will result when the Cre recombinase enters the nucleus.
in which Cre was fused with the estrogen receptor downstream of the Keratin 5 (K5) promoter. K5 is expressed in the bulge region of the hair follicle and basal layer of the epidermis. Stem cells, which are thought to be the targets of chemical carcinogens, reside within the bulge region of the hair follicle. Additionally, K5 is expressed in epithelial cells of the tongue, esophagus, and female urogenital system (Liang, You et al. 2009). We would fuse the Cre recombinase gene to a mutated estrogen receptor that binds tamoxifen instead of estrogen in order to create an inducible model of HuR knockout in the skin, thus providing an additional level of control (Vasioukhin, Degenstein et al. 1999). Our schematic illustrates a proposed model of how to sequester an active Cre-ER in the cytoplasm. The estrogen receptor fused to Cre relocates to the nucleus upon binding to its ligand tamoxifen where it can activate loxP recombination and cause HuR deletion (Figure 7.3). Our model is designed to be both spatially and temporally regulated. The application of topical tamoxifen and the use of the K5CreER construct ensure that we delete HuR only in the skin at specific time points. This model could be used in conjunction with the multi-stage chemical carcinogenesis protocol or the photocarcinogenesis model to provide us with information about the role of HuR during NMSC development in vivo.

7.3 Overall conclusions

We are the first group to report that the enhancement of ODC mRNA stability is a mechanism by which ODC is deregulated during tumor development. In addition, we show here for the first time that ODC is post-transcriptionally regulated by the binding of the trans-acting factor HuR. The cytoplasmic accumulation of the HuR protein during tumor development causes an increase in mRNA stability of the ODC transcript. These data highlight two targets for the chemoprevention and/or chemotherapy of NMSC.
Figure 7.3. Schematic of the Cre-loxP system implemented for conditional HuR knockout in the skin.

The Keratin 5 (K5) promoter which is expressed in the bulge region of the hair follicle and the basal layer of the skin is upstream of the Cre-estrogen receptor (CreER) fusion protein. This fusion protein is localized to the cytoplasm until tamoxifen enters the cytoplasm. Tamoxifen binds to the estrogen receptor and causes Cre-ER to enter the nucleus where Cre activates loxP recombination and HuR exon 2 deletion.
The number of Americans diagnosed with NMSC is on the rise. An estimated 13 million white-non Hispanic Americans account for over 22 million diagnosed BCCs and SCCs, making NMSC a huge public health concern (Stern 2010). These staggering figures in conjunction with a lack of chemoprevention and chemotherapeutic options, highlight the need to study NMSC development. Currently, the best treatment for NMSC is tumor excision, which can lead to extensive scarring. These abysmal statistics are further compounded by the rising healthcare costs for NMSC diagnosis and treatment.

ODC, the first enzyme in the polyamine biosynthetic pathway, has emerged as a contributor of both chemical carcinogenesis-induced and UVB-induced skin cancer development (O'Brien 1976; O'Brien, Megosh et al. 1997; Ahmad, Gilliam et al. 2001; Feith, Shantz et al. 2001). The specific, irreversible inhibitor of ODC, DFMO, has been shown to reduce tumors in mouse models of skin cancer (Peralta Soler, Gilliard et al. 1998; Smith, Trempus et al. 1998; Fischer, Lee et al. 2001). Moreover, it has been involved in numerous chemotherapeutic studies for skin, colon, and breast cancers (Love, Carbone et al. 1993; Love, Jacoby et al. 1998; Fabian, Kimler et al. 2002). However, studies revealed that DFMO was not an effective chemotherapeutic agent in humans when administered as a single agent.

It was not until recently that DFMO re-emerged as a potent chemopreventative agent. As mentioned in Chapter 1, elevated ODC activity and polyamine levels occur in colorectal cancer (Rozhin, Wilson et al. 1984). In APC\textsuperscript{min+} mice, a mouse model of colon cancer in which wild-type alleles of the mouse APC homologue are lost, DFMO has been shown to suppress intestinal tumorigenesis (Erdman, Ignatenko et al. 1999), suggesting that ODC activity has a causal effect on tumorigenesis. In a phase IIb clinical trial, the lowest oral dose of DFMO that was effective in reducing the polyamine content in human rectal mucosa was determined with minimal side effects (Meyskens,
suggesting that DFMO is a safe chemopreventive agent. In a phase IIb/III clinical trial, the combination of DFMO and sulindac, a non-selective inhibitor of both COX-1 and COX-2, was shown to be effective in reducing the number of metachronous colorectal adenomas in patients with a history of adenomas (Meyskens, McLaren et al. 2008). These data demonstrate that DFMO is effective as a chemoprevention agent.

A phase III study of daily DFMO oral intake (0.5 g/m²/day) was conducted for 5 years in patients with a history of NMSC in order to determine if intake of DFMO caused a reduction in NMSC number (Bailey 2010). The studies showed a trend towards the reduction of NMSC in individuals taking DFMO, with a significant reduction in BCCs, suggesting that DFMO has the potential to be a potent chemopreventative agent. Additionally, a study in which DFMO was topically administered to individuals with a history of the SCC precursor, actinic keratosis (AK), demonstrated that DFMO treatment led to a significant decrease in the number of AKs (Alberts, Dorr et al. 2000). These studies support the idea that ODC is an important target for preventing not only NMSC, but other epithelial cancers.

Similar to ODC activity, HuR cytoplasmic accumulation positively correlates with high tumor grade and poor disease outcome for numerous types of cancers, including Merkel cell carcinoma and ovarian cancers (Erkinheimo, Lassus et al. 2003; Koljonen, Bohling et al. 2008). Furthermore, HuR has been shown to stabilize mRNA transcripts associated with tumor development such as COX-2, pro-Tα, and now, ODC (Lal, Kawai et al. 2005; Zhang and Bowden 2008; Nowotarski and Shantz 2010). Thus, inhibiting ODC induction by either DFMO or polyamine analogues, by itself or in conjunction with maintaining nuclear HuR in keratinocytes, could lead to fewer BCCs and SCCs. However, in order to maintain nuclear HuR, we need to elucidate the pathway(s) involved in HuR shuttling in NMSC development. As cytoplasmic HuR binds to and
stabilizes many targets, it is imperative to fully understand the upstream targets which cause HuR localization changes because ablating HuR in cells could produce both off-target and cytotoxic effects. Once the pathway(s) which regulates HuR localization is characterized, we can create therapies which target cytoplasmic HuR specifically in these cancer cells and cause the relocation of HuR from the cytoplasm to the nucleus.

Recently there have been investigations looking at HuR inhibition by low-molecular weight compounds. Using high-throughput screening, the compound MS-444 was shown to bind to HuR and inhibit its ability to bind to mRNA targets. MS-444 was shown to have low toxicity in mice which suggests that HuR inhibition may not affect essential cellular processes in adults (Meisner, Hintersteiner et al. 2007). The safety and efficacy of such compounds are being tested. However, these studies support the idea that HuR is a powerful target for combating not only cancer but inflammation, and provide a tool for developing anti-HuR drugs.

The data provided in this thesis directly implicate HuR cytoplasmic accumulation with subsequent binding to, and stabilization of, the ODC mRNA with NMSC. However, these data may be applicable to other epithelial tumors, as both ODC induction and HuR cytoplasmic accumulation have been demonstrated in other tumors of epithelial origin such as breast, colon, and ovarian cancers (Erkinheimo, Lassus et al. 2003; Lopez de Silanes, Fan et al. 2003). Thus, the data described here provide supporting evidence that cytoplasmic HuR and ODC are targets for the treatment and prevention of these cancers as well. The development of ODC and cytoplasmic HuR specific therapies would therefore be valuable for combating not only NMSC, which is rarely lethal, but also for more lethal epithelial cancers.

Understanding the mechanisms of how ODC and HuR interact during skin tumor development will provide the basic science necessary to create effective pharmacological agents to combat NMSC. The work in this thesis has shown that ODC
RNA and HuR protein interact in a functionally significant manner, thus providing the fundamental knowledge necessary to further understand the complexity of ODC and HuR regulation in both normal and neoplastic cells.
APPENDIX A

M-fold Data

This section displays the most energetically favorable mRNA secondary structures for each of the fragments or deletion constructs used to map the HuR binding site on the ODC 3’UTR. The ΔG values are shown in Table 4.1 and results are interpreted in Chapter 4.
Figure Appendix A.1. The ODC 3'UTR secondary structure as predicted by the M-fold database.

The full length ODC 3'UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. $\Delta G = -226.14$ kcal/mol.
Figure Appendix A.2. The ODC 3’UTR Deletion 1 (bases 1797-2141) secondary structure as predicted by the M-fold database.

The full length ODC 3’UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. ΔG= -91.35 kcal/mol.
The full length ODC 3’UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. ΔG=−46.27 kcal/mol.
Figure Appendix A.4. The ODC 3’UTR Deletion 3 (bases 1797-1850) secondary structure as predicted by the M-fold database.

The full length ODC 3’UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. $\Delta G = -12.90$ kcal/mol.
**Figure Appendix A.5.** The ODC 3'UTR Deletion 4 (bases 1940-2547) secondary structure as predicted by the M-fold database.

The full length ODC 3'UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. ΔG= -185.76 kcal/mol.
The full length ODC 3'UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. ΔG=-127.92 kcal/mol.
Figure Appendix A.7. The ODC 3’UTR Fragment 1 (bases 1797-1950) secondary structure as predicted by the M-fold database.

The full length ODC 3’UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. $\Delta G = -40.27 \text{ kcal/mol}$. 
Figure Appendix A.8. The ODC 3'UTR Fragment 2 (bases 1928-2078) secondary structure as predicted by the M-fold database.

The full length ODC 3'UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. $\Delta G = -42.30$ kcal/mol.
Figure Appendix A.9. The ODC 3’UTR Fragment 3 (bases 2045-2217) secondary structure as predicted by the M-fold database.

The full length ODC 3’UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. ΔG=-39.65 kcal/mol.
Figure Appendix A.10. The ODC 3'UTR Fragment 4 (bases 2195-2369) secondary structure as predicted by the M-fold database.

The full length ODC 3’UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. \( \Delta G = -49.57 \text{ kcal/mol} \).
**Figure Appendix A.11.** The ODC 3’UTR Fragment 5 (bases 2348-2547) secondary structure as predicted by the M-fold database.

The full length ODC 3’UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. $\Delta G=-64.80$ kcal/mol.
Figure Appendix A.12. The 3'UTR of ODC (bases 1851-2151) secondary structure as predicted by the M-fold database.

The full length ODC 3'UTR secondary structure was predicted using the M-fold database. The most energetically favorable secondary structure was chosen. ΔG=−77.05 kcal/mol.
APPENDIX B

Initial characterization of two additional mouse keratinocyte-derived cell lines used to study NMSC promotion

In addition to the experiments described using C5N and A5 cells which represent tumor initiation and progression, we conducted initial characterization experiments on two additional cell lines which are described here. P1 cells were isolated from the benign papillomas of mice that had been treated with DMBA and TPA. B9 cells were isolated from the squamous carcinomas of mice that had been treated with DMBA and TPA (Zoumpourlis, Solakidi et al. 2003).
Figure Appendix B.1. Cell line morphology.

P1 and B9 were derived from mice that had been treated with the DMBA/TPA protocol. P1 cells were isolated from benign papillomas and B9 cells were isolated from squamous cell carcinomas. Both P1 and B9 cells display a classical epithelial cell morphology (Refer to Figure 3.1 for C5N and A5 morphology).
Table Appendix B.1. Generation time of C5N, P1, B9, and A5 cells.

Cells were seeded at a density of 2000 cells per cm$^2$ on a 24 well plate. The number of cells per cm$^2$ was measured at time 0 h (after allowing the cells to adhere to the plates for 16 h). The cell number was measured daily until cells reached the stationary phase. Log of cells per cm$^2$ was plotted against time and the generation times were determined using exponential curve fit analysis. This experiment is a preliminary result, conducted once for the P1 and B9 cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Generation Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5N</td>
<td>24.9</td>
</tr>
<tr>
<td>P1</td>
<td>17.1</td>
</tr>
<tr>
<td>B9</td>
<td>14.1</td>
</tr>
<tr>
<td>A5</td>
<td>19.1</td>
</tr>
</tbody>
</table>
Figure Appendix B.2. ODC enzyme activity during log-phase growth is higher in P1 papilloma cells than in B9 squamous cell carcinomas.

The ODC enzyme activity was measured in all four cell lines at 72 h using the technique described in the Materials and Methods chapter. Values are the means ± S.E. from 10 plates. **P<0.005.
Figure Appendix B.3. ODC transcription is similar between P1 and B9 cells.

The level of ODC transcription was assessed by using the pODCLuc plasmid in which the mouse ODC promoter sequence was placed upstream of the firefly luciferase gene. Luciferase assays were conducted as described in the Materials and Methods chapter. The data were normalized to Renilla luciferase and the pGL3 Control luciferase activity was set to 1 with the pODCLuc luciferase activity measurements being expressed as a percent of the pGL3 Control. Values are means ± S.E. from 12 plates.
Table Appendix B.2. ODC mRNA is more stable in B9 squamous carcinoma cells when compared to P1 benign papilloma cells.

ODC mRNA half-life was determined by adding 5 µg Actinomycin D/ml to each plate. Total RNA was isolated at 0, 4, and 8 h post Actinomycin D treatment, and the levels of ODC and Cyclophilin A mRNA were assessed by Northern blot analysis. Northern blots were probed for ODC and Cyclophilin A simultaneously. The bands were quantitated using Syngene software and the ODC mRNA data were normalized to the Cyclophilin A control. The data represent a preliminary experiment performed once, therefore statistics cannot be applied (Work done by Suzanne Sass-Kuhn for P1 and B9 cells).
Figure Appendix B.4. HuR localizes mainly to the nucleus in P1 cells and is both nuclear and cytoplasmic in B9 cells.

P1 and B9 cells were seeded onto glass coverslips coated with poly-L-lysine at a density of 50,000 cells per 6 well plate. Upon 70% confluency, HuR protein was detected using anti-HuR antibody and Cy-2 secondary antibody as described in the Materials and Methods chapter. Images were taken at 40X magnification. Representative images are shown. This experiment was done in duplicate with reproducible results (Refer to Figure 4.8 for C5N and A5 pictures).
Literature Cited


Hecker, E. (1971). "Isolation and characterization of the co-carcinogenic principles from croton oil." Methods Cancer Res. 6: 439-484


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Honors and Awards

Graduate Alumni Endowment Scholarship, The Pennsylvania State University College of Medicine, (2010)
Morgan Travel Award, The Pennsylvania State University College of Medicine, (2010)
Outstanding Poster, The Pennsylvania State University College of Medicine, (2009)
Graham Fellowship, The Pennsylvania State University College of Medicine, (2005-2007)