CYTOPLASMIC ACCUMULATION OF THE RNA BINDING PROTEIN HUR STABILIZES THE ORNITHINE DECARBOXYLASE TRANSCRIPT IN A MURINE NON-MELANOMA SKIN CANCER MODEL*

Shannon L. Nowotarski and Lisa M. Shantz
From Department of Cellular and Molecular Physiology, Penn State College of Medicine, Hershey, PA 17033

Running Title: HuR binding stabilizes ODC RNA in skin cancer
Address correspondence to: Lisa M. Shantz, Ph.D., The Penn State College of Medicine, Department of Cellular and Molecular Physiology, Room C4731, 500 University Drive, Hershey, PA 17033. Tel: 717-531-1562; Fax: 717-531-7667; E-mail: lms17@psu.edu.

Ornithine decarboxylase (ODC) catalyzes the conversion of the amino acid ornithine to the diamine putrescine, which is subsequently converted to the higher polyamines spermidine and spermine (1,2). Polyamines are small, ubiquitous polycations, and are necessary for normal cell growth and development. Thus, the ablation of ODC is lethal in utero (3). Due to their charge, polyamines are able to bind to RNA, DNA, and proteins and thereby influence gene expression (4). 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 (5-10). A link between neoplastic transformation and increased ODC enzyme activity, as well as increases in intracellular putrescine and spermidine, have been well documented in animal models of skin carcinogenesis and other epithelial tumors (11-17). Moreover, a link between human non-melanoma skin cancer (NMSC), the most prevalent cancer in the United States, and elevated ODC enzyme activity levels has been described (18). The induction of ODC activity during tumor development has been attributed to both increased Odc gene transcription and translation of the ODC mRNA (9,19).

Interestingly, changes in intracellular polyamine pools have also been shown to affect the localization of RNA binding proteins (RBPs), which influence the stability of their target mRNA transcripts (20-22). Control of mRNA decay is a rapid means of regulating the expression of mRNAs (23). RBPs bind to adenosine- and uracil-rich elements (AREs), sequences classically located within the 3'UTR of labile mRNAs (24). These sequences behave as cis-acting elements, and are located in numerous proto-oncogene, cytokine, and transcription factor mRNAs (25). Three categories of RBPs have been defined: those that stabilize mRNA, those that destabilize...
mRNA, and those that can both stabilize and destabilize mRNA (26). Additionally, it has been shown that some RBPs can alter the translational efficiency of their bound RNA transcript (27,28).

Human antigen R (HuR) belongs to the family of Hu proteins that are highly homologous to the embryonic lethal abnormal vision (ELAV) family of proteins. Unlike its family members HuB, HuC, and HuD, which are expressed exclusively in neurons, HuR is ubiquitously expressed (28). The HuR protein contains three RNA recognition motifs (RRMs) (29). RRM 1 and RRM2 have been shown to recognize AREs located within the mRNA, while it has been suggested that RRM3 recognizes the poly(A) tail of the mRNA transcript (29). 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 (30). 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 H2O2 (31,32). In the cytoplasm, HuR can stabilize and/or increase the translational efficiency of its bound mRNA transcript (29). Interestingly, a correlation between HuR localization and neoplastic transformation has been described. HuR has been shown to bind to and stabilize numerous transcripts whose overexpression contribute to tumorigenesis, such as TSP1, COX2, and ProTα (31,33,34). The studies described here support the hypothesis that HuR plays a major role in the regulation of ODC mRNA decay in skin tumor development.

The mouse keratinocyte cell model used in these studies is comprised of two cell lines: C5N cells have a wild-type keratinocyte morphology and contain no detectable mutations in the H-ras gene, which is mutated at a high frequency in NMSC. A5 cells have a spindle carcinoma phenotype and were isolated from the tumor of a mouse that had been subjected to the classical two-stage protocol of initiation and promotion (35). Here we show that ODC enzyme activity is induced and ODC mRNA is markedly stabilized 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.

**EXPERIMENTAL PROCEDURES**

**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. These cells have been described previously (35). Passages 5-20 were used in the experiments. Stock flasks were incubated at 37°C in a humidified atmosphere of 95% air/5% CO2.

**Assay for ODC Enzyme Activity**—ODC enzyme activity was determined by radiometric assay measuring the amount of 14CO2 released from each reaction in which L-[1-14C]-ornithine is enzymatically converted to 14CO2 by ornithine decarboxylase. Collection and analysis were carried out as described (36).

**RNA Extraction, Northern Blot Analysis, and Actinomycin D Experiments**—RNA was extracted using the TRIzol reagent according to the manufacturer’s instructions (Invitrogen). For Northern blot analysis 20 µg RNA was loaded onto a 1.2% (w/v) agarose-formaldehyde gel and 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 32P labeled cDNA probes synthesized for ODC and cyclophilin A. The primers are listed in Supplemental Data Table 1. The bands were quantitated using Syngene Software (Syngene, Frederick, MD) and the ODC mRNA data was 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.
mRNA half-life was determined using curve fit analysis.

**Real-Time PCR Analysis**-Total RNA was isolated as described above and used in reverse transcription and PCR amplification. 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. Analysis was performed by using the Step One Plus Real-Time PCR software (Applied Biosystems). The data were normalized to Cyclophilin A.

**Western Blotting**-Western blotting was conducted as described previously (37). To prepare cytoplasmic and nuclear extracts, cells were fractionated using the Pierce Fractionation Kit as per the manufacturer’s instructions (Pierce, Rockford, IL). To check the extracts for nuclear contamination, Western blot analysis was conducted on 20 µg of extract and the alpha/beta Tubulin and Histone H3 antibodies were used. The antibodies recognizing HuR (Santa Cruz Biotechnology, Santa Cruz, CA), GAPDH (BioDesign International, Saco, ME), Histone H3, and alpha/beta Tubulin (Cell Signalling, Danvers, MA) were all used at a 1:1000 dilution.

**Preparation of Synthetic RNA Transcripts**-cDNA from C5N and A5 cells was used as a template for PCR amplification of the 5'UTR, coding region (CR), and 3'UTR of ODC. The sense primers for each reaction contained the T7 RNA polymerase promoter sequence on the 5' end and are listed in Supplemental Data Table 1. The PCR templates were used to transcribe biotinylated RNAs by using the T7 polymerase and biotin-labeled 14-CTP (Invitrogen) as reported previously (22). In order to elucidate the binding site of HuR on the 3'UTR of ODC, various fragment and deletion constructs were created using sense primers containing the T7 promoter sequence and antisense primers that created varying length fragments of the ODC 3'UTR. GAPDH and c-Myc were utilized as negative and positive controls respectively. All primers used are listed in Supplemental Data Table 1.

**Biotin-labeled RNA-Protein Binding Assay**-The biotin-labeled RNA transcripts (15 µl) were incubated with 120 µg of cytoplasmic lysate for 30 min at room temperature. The RNA-protein complexes were then harvested by using paramagnetic streptavidin-conjugated Dynabeads (Invitrogen), and were analyzed by Western blotting using the HuR antibody described above.

**RNA Protein-binding Assays**-For IP of endogenous RNA-protein binding complexes, 2000 µg of cytoplasmic lysate was incubated for 2 h at room temperature with 50% (v/v) suspension of Protein A-Sepharose beads (Sigma-Aldrich, St. Louis, MO) that had been pre-coated with 30 µg of either mouse IgG (Invitrogen) or HuR antibodies as described (38). The RNA IP material was analyzed by washing the beads with NT-2 buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl₂ and 0.05% Nonidet P-40), and further incubated with 0.5 mg/ml Proteinase K, NT-2 buffer, and 0.1% SDS for 15 min at 55°C. RNA was precipitated using phenol-chloroform extraction in the presence of glycolblue (Qiagen, Valencia, CA). RNA from the IP was then reverse transcribed to detect the presence of ODC mRNA. The primers used for the ODC coding region are listed in Supplemental Data Table 1.

**Immunofluorescence Staining**-A5 and C5N cells were seeded onto glass coverslips that had been pre-coated with poly-L-lysine (Sigma-Aldrich). Cells were allowed to grow to 70% confluency and were then fixed in 50/50 acetone/methanol solution at -20°C for 5 min. The samples were incubated overnight at 4°C 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 coverslips 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., Melville, NY). Images were processed using Windows Photo Gallery (Microsoft, Redmond, WA).

**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’ GGUUGAAUC
UGCAAGCUU 3’. For RNAi experiments, C5N and A5 cells were allowed to grow to 70% confluency and transfected with the siRNA (100 nM final concentration) specific for the HuR coding region or a negative control according to the manufacturer’s instructions. Mock transfected cells were treated with the transfecting agent DharmaFECT 1 (Thermoscientific) only. Knockdown efficiency was assessed 72 h post-transfection through RT-PCR, Northern blot, or Western blot analysis. For Northern blot analysis and RT-PCR, RNA was harvested using methods described above. For Western blot analysis protein was harvested in 1X 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) (Cell Signaling Technology). ODC enzyme activity was tested in mock, negative control, or siHuR treated cells as described above. ODC mRNA stability in mock, negative control, or siHuR treated cells was also assessed by treating each sample with 10 µg/ml of Actinomycin D. RNA was harvested at 0 h, 4 h and 8 h, and ODC half-life was determined as described above.

**RESULTS**

**ODC enzyme activity and mRNA stability are increased in transformed compared to non-transformed keratinocyte-derived cells lines**- ODC enzyme activity assays revealed a 4-fold change in ODC enzyme activity between the two cell lines (Figure 1A), with C5N keratinocytes displaying low ODC enzyme activity while the A5 cells exhibited a significant induction of ODC enzyme activity. The upregulation of ODC enzyme activity was not due to an increase in transcription as qRT-PCR revealed no significant difference in steady-state mRNA levels between the two cell lines (Figure 1B). Using both Northern blot analysis with subsequent band quantitation and qRT-PCR, we determined that ODC mRNA half-life was 4 h in the C5N keratinocytes compared to 15 h in A5 cells (Figure 1C). 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.

**HuR is able to bind to the ODC 3’UTR in A5 spindle carcinoma cells, but not C5N keratinocytes**- Previous results have shown that the intracellular polyamine pool can affect the localization of the stabilizing RBP HuR in IEC-6 cells (21,22). HuR has been shown to preferentially bind to ARE sequences that are located in the 3’UTR of a target mRNA transcript (28,39). Upon examination of the mouse 3’UTR for ODC, one classical AUUUA ARE was located (Figure 2A). 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. The data show that cytoplasmic extracts of C5N cells demonstrate no measurable binding of HuR to either the ODC 3’UTR or the ODC coding region. Conversely, cytoplasmic A5 extracts exhibit strong HuR binding to the ODC 3’UTR biotinylated probe (Figure 2B, 2C). 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 2D). 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.

In order to determine the location of the HuR binding site in the 3′UTR of ODC, we created overlapping biotinylated probes of approximately 150-200 base pairs in length that scanned the entire ODC 3′UTR (Figure 3A). A5 cytoplasmic lysates were used to perform these mapping experiments because we did not observe HuR binding from C5N lysates (Figure 2C). The association of HuR protein with a synthetic ODC mRNA transcript was detected by Western blot analysis of the pull-down material (Figure 3B). The ODC full length 3′UTR transcript associated with HuR. 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) also 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 (40). 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 base pairs in length (Figure 4A). 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 4B). 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 4C).

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. In agreement with the biotinylated pulldown data, little or no 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 5A and 5B). 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 can act as a partial contaminant in IP materials (41). The positive control c-Myc mRNA associated strongly with HuR protein in A5 lysates (Figure 5A) (40).

HuR cellular localization differs between normal mouse keratinocytes and skin tumor-derived cells-The results above show strong binding of HuR to ODC mRNA in transformed A5 cells, but little or no association in C5N keratinocytes, despite having similar levels of HuR protein as seen in the whole-cell lysates of each cell line (Figure 2D). To address the issue of preferential binding of 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 (data not shown). Given that the ODC 3′UTR was not mutated in the A5 cells, another possible explanation is that the two cell lines display different cellular localizations of the HuR protein. HuR has been shown to shuttle between the nucleus and cytoplasm in response to various stimuli or intrinsic cellular conditions. Additionally, cytoplasmic HuR in human tumors correlates with an increased tumor grade and a decreased survival rate (42,43). 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 6). These data affirm our previous findings in Figures 2 and 5, as both the biotinylated pulldown assay and mRNP assay use cytoplasmic extract. This difference in localization likely accounts for HuR’s inability to bind to ODC mRNA in C5N cytoplasmic extracts. In fact, when C5N nuclear extracts were used to investigate HuR binding to the ODC mRNA transcript, we show that HuR is able to bind to the ODC 3′UTR (Figure 7A). The C5N nuclear extract also displayed lower-level binding to the 5′UTR and coding region of the ODC mRNA transcript (Figure 7B).
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 assessed by qRT-PCR (Figure 8A). 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 8B and 8C).

To rule out toxicity, the cell viability of A5 cells treated with siHuR and the negative control siRNA was assessed. No change in cell viability was measured in siHuR treated cells when compared to either untreated or negative control siRNA treated cells (Figure 8D). These data affirm that siHuR treatment in A5 cells is not toxic.

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 8E). 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 8F). These findings strongly suggest that HuR enhances the stability of the ODC mRNA transcript in mouse keratinocyte cells through direct 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 decrease 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 cells express HuR almost exclusively in the nucleus (Figure 6), 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 9A). In fact, HuR protein levels decreased by more than 50 percent in siHuR treated cells when compared to cells that had been treated with the negative control siRNA (Figure 9B). However, the knockdown of HuR had no effect on the ODC mRNA stability in C5N cells. Both siHuR treated and negative control siRNA treated cells had the same ODC mRNA half-life of approximately 9 h (Figure 9C). Correspondingly, no change in ODC enzyme activity was observed in siHuR treated C5N cells when compared to the negative control siRNA treated cells (data not shown).

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 also saw a stabilization of ODC mRNA in A5 cells treated with the same negative control siRNA (compare Figure 1C with Figure 8F). In any case, we show that ODC mRNA stability is unchanged in C5N cells treated with siHuR and that A5 cells treated with siHuR demonstrate a reduction in both ODC mRNA stability and ODC enzyme activity. 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.

**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 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 contained within the 3'UTR of the ODC mRNA transcript (Figures 1-4). Data from deletion constructs indicate that HuR may bind to multiple sites within the ODC 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 (44).
Furthermore, Yeap et al. demonstrated HuR binding to UC-rich motifs in the 3’UTR of Androgen Receptor mRNA (45). The general area of strongest HuR binding on the ODC 3’UTR, between bases 1851 and 2151, is also U-rich (Figure 4C).

The results presented here show that HuR binds to the ODC 3’UTR of A5 spindle carcinoma cells, but no association was demonstrated in C5N keratinocytes (Figure 2C and Figure 5). The binding status of HuR to 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 6). This phenomenon is in accordance with previous results showing that the cytoplasmic localization of HuR stabilizes p53 and nucleophosmin (22).

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 (42,46,47). C5N nuclear extracts, which have an abundance of HuR protein, demonstrate binding to a synthetic ODC 3’UTR transcript (Figure 7). Given that the sequences of the ODC 3’UTR are identical in both cell lines, these data suggest that HuR localization dictates whether the HuR protein binds to and stabilizes the ODC transcript.

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 (21). Furthermore, the depletion of the polyamine intracellular pool through the inhibition of ODC was shown to cause an increase in HuR cytoplasmic presence and an increase in ATF-2 mRNA stability (20). In contrast, we do not see HuR shuttle to 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 (data not shown), 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 (31,48,49).

We note in A5 spindle carcinoma cells that both ODC enzyme activity and ODC mRNA half-life are upregulated (Figure 1A and Figure 1C). 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 in A5 cells, thereby causing its cytoplasmic accumulation, where it can bind to a variety of target mRNAs, including ODC (50).

We are currently investigating upstream targets that are activated in these cells and that may cause this localization change in HuR between our spindle carcinoma cells and normal keratinocytes. 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 (51).

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 (52). ODC has been shown to be both necessary and sufficient for the onset of NMSC in mice, and is also elevated in human NMSC (12,14,53-55). 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 α-difluoromethylornithine (DFMO) a suicide inactivator of ODC, markedly reduces the incidence of new basal cell carcinoma in subjects with a history of skin cancer (18). Earlier studies showed that topical DFMO administration reduced the incidence of actinic keratosis, a precursor of squamous cell carcinoma, in subjects at high risk for NMSC (56). The studies provided here shed more light on 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 subsequently 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 stabilization of the ODC transcript is attributed 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 in HuR binding status to the ODC 3’UTR between the two cell lines. Furthermore, we provide a functional link between HuR and ODC using RNA interference to knockdown HuR, resulting in the destabilization of the ODC message and a decrease in ODC activity in carcinoma cells but not in normal keratinocytes. These findings not only demonstrate a novel mechanism of ODC regulation, but also provide another potential target for chemoprevention and chemotherapeutics for NMSC.

REFERENCES

1. Wallace, H. M., Fraser, A. V., and Hughes, A. (2003) *Biochem J* **376**, 1-14
2. Pegg, A. E. (2006) *J Biol Chem* **281**, 14529-14532
3. Pendeville, H., Carpino, N., Marine, J. C., Takahashi, Y., Muller, M., Martial, J. A., and Cleveland, J. L. (2001) *Mol Cell Biol* **21**, 6549-6558
4. Wallace, H. M. (2000) *Eur J Clin Invest* **30**, 1-3
5. Bello-Fernandez, C., Packham, G., and Cleveland, J. L. (1993) *Proc Natl Acad Sci USA* **90**, 7804-7808
6. Zhao, B., and Butler, A. P. (2001) *Mol Carcinog* **32**, 92-99
7. Zhao, B., Kumar, A. P., and Butler, A. P. (2000) *Mol Carcinog* **29**, 212-218
8. Shantz, L. M., and Pegg, A. E. (1999) *Int J Biochem Cell Biol* **31**, 107-122
9. Shantz, L. M. (2004) *Biochem J* **377**, 257-264
10. Wallon, U. M., Persson, L., and Heby, O. (1995) *Mol Cell Biochem* **146**, 39-44
11. Feith, D. J., Bol, D. K., Carboni, J. M., Lynch, M. J., Sass-Kuhn, S., Shoop, P. L., and Shantz, L. M. (2005) *Cancer Res* **65**, 572-578
12. O'Brien, T. G., Megosh, L. C., Gilliard, G., and Soler, A. P. (1997) *Cancer Res* **57**, 2630-2637
13. O'Brien, T. G. (1976) *Cancer Res* **36**, 2644-2653
14. Smith, M. K., Trembus, C. S., and Gilmour, S. K. (1998) *Carcinogenesis* **19**, 1409-1415
15. Gerner, E. W., and Meyskens, F. L., Jr. (2004) *Nat Rev Cancer* **4**, 781-792
16. Auvinen, M., Paasinen, A., Andersson, L. C., and Holta, E. (1992) *Nature* **360**, 355-358
17. Peralta Soler, A., Gilliard, G., Megosh, L., George, K., and O'Brien, T. G. (1998) *Cancer Res* **58**, 1654-1659
18. Bailey, H. H., Kim, K., Verma, A. K., Sielaff, K., Larson, P. O., Snow, S., Lenaghan, T., Viner, J. L., Douglass, J., Dreckschmidt, N. E., Hamielec, M., Pomplun, M., Sharata, H. H., Puchalsky, D., Berg, E. R., Havighurst, T. C., and Carbone, P. P. *Cancer Prev Res (Philad Pa)* **3**, 35-47
19. Mimori, K., Mori, M., Shiraiishi, T., Tanaka, S., Haraguchi, M., Ueo, H., Shirasaka, C., and Akiyoshi, T. (1998) *Int J Oncol* **12**, 597-601
20. Xiao, L., Rao, J. N., Zou, T., Liu, L., Marasa, B. S., Chen, J., Turner, D. J., Zhou, H., Gorospe, M., and Wang, J. Y. (2007) *Mol Biol Cell* **18**, 4579-4590
21. Zou, T., Liu, L., Rao, J. N., Marasa, B. S., Chen, J., Xiao, L., Zhou, H., Gorospe, M., and Wang, J. Y. (2008) *Biochem J* **409**, 389-398
22. Zou, T., Mazan-Mamczarz, K., Rao, J. N., Liu, L., Marasa, B. S., Zhang, A. H., Xiao, L., Pullmann, R., Gorospe, M., and Wang, J. Y. (2006) *J Biol Chem* **281**, 19387-19394
23. Dreyfuss, G., Kim, V. N., and Kataoka, N. (2002) *Nat Rev Mol Cell Biol* **3**, 195-205
24. Parker, R., and Sheth, U. (2007) *Mol Cell* **25**, 635-646
25. Audic, Y., and Hartley, R. S. (2004) *Biol Cell* **96**, 479-498
26. Guhaniyogi, J. a. G. B. (2001) *Gene* **265**, 11-23
27. Mazan-Mamczarz, K., Galban, S., Lopez de Silanes, I., Martindale, J. L., Atasoy, U., Keene, J. D., and Gorospe, M. (2003) *Proc Natl Acad Sci U S A* **100**, 8354-8359
28. Hinman, M. N., and Lou, H. (2008) *Cell Mol Life Sci* **65**, 3168-3181
29. Brennan, C. M., and Steitz, J. A. (2001) *Cell Mol Life Sci* **58**, 266-277
30. Fan, X. C., and Steitz, J. A. (2008) *Cell Mol Life Sci* **65**, 3168-3181
31. Zhang, J., and Bowden, G. T. (2008) *Mol Carcinog* **32**.
32. Kuwano, Y., Kim, H. H., Abdelmohsen, K., Pullmann, R., Jr., Martindale, J. L., Yang, X., and Gorospe, M. (2008) *Mol Cell Biol* **28**, 4562-4575
33. Mazan-Mamczarz, K., Hagner, P. R., Corl, S., Srikantan, S., Wood, W. H., Becker, K. G., Gorospe, M., Keene, J. D., Levenson, A. S., and Gartenhaus, R. B. (2008) *Oncogene* **34**.
34. Lal, A., Kawai, T., Yang, X., Mazan-Mamczarz, K., and Gorospe, M. (2005) *Embo J* **24**, 1852-1862
35. Zoumpourlis, V., Solakidi, S., Papathoma, A., and Papaevangeliou, D. (2003) *Carcinogenesis* **24**, 1159-1165
36. Coleman, C. S. a. P., A.E. (ed) (1998) *Assay of mammalian ornithine decarboxylase activity using [14C]-ornithine*, Vol. 79, Humana Press Inc., Totowa, NJ
37. Origanti, S., and Shantz, L. M. (2007) *Cancer Res* **67**, 4834-4842
38. Lal, A., Mazan-Mamczarz, K., Kawai, T., Yang, X., Martindale, J. L., and Gorospe, M. (2004) *Embo J* **23**, 3092-3102
39. Ma, W. J., Cheng, S., Campbell, C., Wright, A., and Furneaux, H. (1996) *J Biol Chem* **271**, 8144-8151
40. Liu, L., Rao, J. N., Zou, T., Xiao, L., Wang, P. Y., Turner, D. J., Gorospe, M., and Wang, J. Y. (2009) *Mol Biol Cell*
41. Abdelmohsen, K., Pullmann, R., Jr., Lal, A., Kim, H. H., Galban, S., Yang, X., Blethrow, J. D., Walker, M., Shubert, J., Gillespie, D. A., Furneaux, H., and Gorospe, M. (2007) *Mol Cell* **25**, 543-557
42. Heinonen, M., Bono, P., Narko, K., Chang, S. H., Lundin, J., Joensuu, H., Furneaux, H., Hla, T., Haglund, C., and Ristimaki, A. (2005) *Cancer Res* **65**, 2157-2161
43. Blaxall, B. C., Dwyer-Nield, L. D., Bauer, A. K., Bohlmeyer, T. J., Malkinson, A. M., and Port, J. D. (2000) *Mol Carcinog* **28**, 76-83
44. Lopez de Silanes, I., Zhan, M., Lal, A., Yang, X., and Gorospe, M. (2004) *Proc Natl Acad Sci U S A* **101**, 2987-2992
45. Yeap, B. B., Voon, D. C., Vivian, J. P., McCulloch, R. K., Thomson, A. M., Giles, K. M., Czyyz-Krzeska, M. F., Furneaux, H., Wilce, M. C., Wilce, J. A., and Leedman, P. J. (2002) *J Biol Chem* **277**, 27183-27192
46. Koljonen, V., Bohling, T., Haglund, C., and Ristimaki, A. (2008) *J Cutan Pathol* **35**, 10-14
47. Erkinheimo, T. L., Lassus, H., Sivula, A., Sengupta, S., Furneaux, H., Hla, T., Haglund, C., Butzow, R., and Ristimaki, A. (2003) *Cancer Res* **63**, 7591-7594
48. Atasoy, U., Watson, J., Patel, D., and Keene, J. D. (1998) *J Cell Sci* **111 ( Pt 21)**, 3145-3156
49. Kim, H. H., and Gorospe, M. (2008) *Cell Cycle* **7**, 3124-3126
50. Lopez de Silanes, I., Lal, A., and Gorospe, M. (2005) *RNA Biol* **2**, 11-13
51. Kim, H. H., Yang, X., Kuwano, Y., and Gorospe, M. (2008) *Cell Cycle* **7**, 3371-3377
52. Bickers, D. R., Lim, H. W., Margolis, D., Weinstock, M. A., Goodman, C., Faulkner, E., Gould, C., Gemen, E., and Dall, T. (2006) *J Am Acad Dermatol* **55**, 490-500
53. Elmet, C. A., and Athar, M. *Cancer Prev Res (Phila Pa)* **3**, 8-11
54. Feith, D. J., Shantz, L. M., and Pegg, A. E. (2001) *Cancer Res* **61**, 6073-6081
55. Gilmore, S. K. (2007) *Toxicol Appl Pharmacol* **224**, 249-256
56. Einspahr, J. J., Nelson, M. A., Saboda, K., Warneke, J., Bowden, G. T., and Alberts, D. S. (2002) *Clin Cancer Res* **8**, 149-155
FOOTNOTES

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Abbreviations used are: NMSC, non-melanoma skin cancer; ODC, ornithine decarboxylase; RBP, RNA binding protein; ARE, adenosine- and uracil-rich elements; HuR, human antigen R; RRM, RNA recognition motif; DFMO, α-difluoromethylornithine

FIGURE LEGENDS

FIGURE 1. ODC enzyme activity and RNA stability are increased in transformed mouse keratinocyte-derived cells when compared to wild-type keratinocytes. A, ODC enzyme activity was determined in C5N cells and A5 cells as described in the Experimental Procedures. Values are the means ± S.E. from 10 plates. *P<0.005 when comparing the two cell lines. B, ODC mRNA steady-state levels were determined by Northern blot analysis as described in the Experimental Procedures. The results were validated using qRT-PCR. Values are means ± S.E. from 4 separate experiments. C, ODC mRNA half-life was determined using 5 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 as described. The results were verified by qRT-PCR. Values are the means ± S.E. from 4 separate experiments.

FIGURE 2. Cytoplasmic HuR from A5 spindle cell carcinoma cells but not C5N keratinocytes is able to bind to synthetic ODC mRNA transcripts. A, Schematic representation of ODC mRNA, showing the putative ARE (gray highlight). B, Western blot analysis to check cytoplasmic extracts for purity. C5N and A5 cells were fractionated as described in the Experimental Procedures. To check the extracts for nuclear contamination, Western blots for alpha/beta Tubulin (cytoplasmic marker) and Histone H3 (nuclear marker) were used. C, To assess HuR binding, cytoplasmic lysates (120 µg) prepared from C5N and A5 cell lines were incubated with 15 µl of synthetic biotin-labeled ODC 3’UTR or ODC coding region (CR) transcript for 30 min at room temperature. The resulting RNP complexes were pulled down using streptavidin beads, and the presence of HuR was assessed via Western blot analysis. This experiment was done in duplicate with similar results. D, Whole-cell lysates were prepared in 1X RIPA buffer. HuR was detected via Western blot analysis. The blot was then stripped and re-probed for GAPDH to confirm equal loading of protein. This experiment was conducted three times with similar results.

FIGURE 3. HuR binds to the proximal region of the ODC 3’UTR. A, Schematic representation of the ODC 3’UTR sequences used for mapping the HuR binding site(s). Five overlapping sequences of approximately equal size were created using the PCR primers described in Supplemental Data Table 1. The PCR product was then biotin-labeled as described in the Experimental Procedures. B, To determine the association of HuR, A5 cytoplasmic lysate (120 µg) was incubated with 15 µl of each biotin-labeled synthetic probe and Western blot analysis for HuR was performed. The full length 3’UTR (FL) was used as a positive control, as was a c-Myc 3’UTR. A GAPDH (Gpdh) biotinylated probe was used as a negative control. Whole-cell lysate (WC) is also shown. This experiment was done in duplicate with similar results.
FIGURE 4. HuR binds between base pairs 1850 and 2151 on the ODC 3’UTR. A, Schematic representation of the ODC 3’UTR deletion constructs used for mapping the HuR binding site(s). Five deletions constructs from both the 3’end and the 5’end of the 3’UTR of ODC ranging in size from 50-500 base pairs in length were created and biotin-labeled as described in the Experimental Procedures. The full length 3’UTR was used as a positive control. B, A representative HuR immunoblot using A5 cytoplasmic lysate and the ODC 3’UTR biotinylated deletion construct probes. This experiment was done in duplicate with reproducible results. C, Schematic representation of the ODC mRNA transcript showing the 3’UTR with the predicted HuR binding region highlighted in gray.

FIGURE 5. Association of endogenous HuR with endogenous ODC mRNA in A5 cells but not in C5N cells. A, Cytoplasmic lysates from C5N and A5 cells were used for immunoprecipitation (IP) with anti-HuR or nonspecific mouse IgG. RNA in the IP material was extracted using phenol chloroform extraction, used in a PCR reaction, and visualized on a 2% agarose gel. The levels of ODC mRNA and GAPDH mRNA were assessed by PCR using primers that were specific for their respective coding regions. The level of c-Myc bound to HuR was assessed as a positive control. This experiment was conducted in triplicate with reproducible results. B, The above experiment was repeated and quantitated using qRT-PCR. ODC mRNA values were normalized to Cyclophilin A. Values are the means ± S.E. of data from 2 separate experiments (n=6). *P<0.005.

FIGURE 6. HuR is exclusively nuclear in C5N normal keratinocytes but is both nuclear and cytoplasmic in A5 spindle carcinoma cells. Cells were grown on glass coverslips as described in the Experimental Procedures. 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 7. Nuclear extracts from C5N keratinocytes display HuR binding to the ODC mRNA transcript. A, A biotinylated pulldown assay using C5N nuclear extracts was conducted as described in the Experimental Procedures. 120 µg of pulldown material as well as 20 µg C5N whole cell lysate were loaded onto a 10% SDS-PAGE gel and HuR binding was assessed via Western blot analysis. A representative immunoblot is shown. This experiment was done in triplicate with similar results. B, The immunoblot described in A 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 quantitation are reported as a percent of the 5’UTR band.

FIGURE 8. HuR silencing decreases both ODC enzyme activity and the stability of ODC mRNA in A5 spindle carcinoma cells. A, Confirmation of HuR knockdown. A5 cells were transfected with a validated siHuR oligonucleotide or mock transfected as described in the Experimental Procedures. 72 h after transfection, cells were harvested, RNA was extracted, and the level of HuR RNA was measured using qRT-PCR. Values are shown as percent HuR when compared to the mock ± S.E. (n=3). B, Western blot analysis to detect HuR protein knockdown. A5 cells were grown and transfected with siRNA duplexes specific for the HuR coding region or a negative control siRNA (denoted as siHuR and Ctrl siRNA respectively). Mock transfected cells (Mock) and untreated A5 cells (WC) are also shown. Protein was harvested after 72 h in 1X RIPA buffer. The level of HuR was measured by Western blot analysis. Equal loading was monitored by GAPDH immunoblotting. C, Quantitation of the Western blot analysis from Panel B. All HuR bands were normalized to GAPDH. A representative blot and band quantitation are shown. D, Cell viability does not change in A5 cells treated with siHuR. Cell viability was measured in untreated A5 cells, mock transfected cells (DharmaFECT1 only), siHuR treated cells, and negative control siRNA (Ctrl siRNA) treated cells using methods described in the Experimental Procedures. The OD measurements for the untreated A5 cells were set to 1. The mock, siHuR, and negative control siRNA treated cells are reported as a mean percent of the untreated cells OD readings ±
E. ODC enzyme activity decreases with HuR knockdown in A5 cells. At 72 h post-siHuR transfection, cells were harvested in ODC harvest buffer, and ODC enzyme activity was assayed as described in the Experimental Procedures. The values are shown as the percent ODC enzyme activity with the mock control cells representing 100% activity. The values are the mean activity ± S.E. These experiments were done in triplicate (n=8). *P<0.005. F. ODC RNA half-life decreases in the presence of siHuR. At 72 h post-transfection, 11 µg Actinomycin D was added to the cell medium, and total cellular RNA was extracted at 0 h, 4 h, and 8 h post-Actinomycin D treatment. The levels of ODC and Cyclophilin A mRNA were measured at each time point using Northern blot analysis, with confirmation by qRT-PCR. ODC levels were normalized to the Cyclophilin A control for each time point. The values are the means ± S.E. (n=3-6).

Figure 9. HuR knockdown in C5N cells does not effect ODC mRNA stability. A. Western blot analysis to detect HuR knockdown. C5N cells were grown and transfected with siRNA duplexes specific for the HuR coding region or a negative control siRNA (denoted as siHuR and Ctrl siRNA respectively). Protein was harvested after 72 h in 1X RIPA buffer. The level of HuR was measured by Western blot analysis. Equal loading was monitored by GAPDH immunoblotting. This experiment was done in triplicate with reproducible results. A representative blot is shown. B. Quantitation of the Western blot analysis from Panel A. All HuR bands were normalized to GAPDH. The negative control siRNA treated cells band (Ctrl siRNA) is set to 1 and the siHuR treated band is reported as a percent of the control siRNA band. A band quantitation is shown. C. ODC mRNA half-life does not change in the presence of siHuR in C5N keratinocytes. At 72 h post-transfection, 11 µg Actinomycin D was added to the cell medium and total cellular RNA was extracted at 0 h, 4 h and 8 h post-Actinomycin D treatment. The levels of ODC and Cyclophilin A mRNA were measured at each time point using qRT-PCR. ODC levels were normalized to the Cyclophilin A control for each time point. The values are the means ± S.E. (n=6).
Figure 1.
Figure 2.
A.  

|   | Full Length 3'UTR (725 bp) |
|---|---------------------------|
| FL |                           |
| F1 | 1797-1950                 |
| F2 | 1928-2078                 |
| F3 | 2045-2217                 |
| F4 | 2195-2369                 |
| F5 | 2348-2547                 |

B.  

| ODC 3'UTR | FL | F1 | F2 | F3 | F4 | F5 | c-myc | Gpdh | WC |
|-----------|----|----|----|----|----|----|-------|------|----|

![Image with bands for HuR (36 kD) and GAPDH (37 kD)]

**Figure 3.**
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Cytoplasmic accumulation of the RNA binding protein HuR stabilizes the ornithine decarboxylase transcript in a murine non-melanoma skin cancer model
Shannon L. Nowotarski and Lisa M. Shantz

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