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14. ABSTRACT

Posttranscriptional control of gene expression is particularly important for oncoproteins and cell cycle proteins because their sustained synthesis favors cell growth rather than differentiation, a hallmark of the neoplastic phenotype. Control is exerted via the opposing actions of the RNA-binding proteins AUF1 and HuR. AUF1 triggers degradation of mRNA subsets while HuR promotes mRNA stabilization. Phase I of this work is to examine the effects of AUF1 and HuR expression levels on global gene expression in human breast carcinoma cells. Phase II is to assess roles of AUF1 and HuR in cellular proliferation and tumorigenesis in vivo. During this funding period, we discovered that AUF1 knockdown accelerates breast cancer cell proliferation and may convert cells to a highly metastatic state. Moreover, AUF1 knockdown elevates expression of the c-myc proto-oncogene, consistent with accelerated proliferation. mRNP immunoprecipitation and RT-PCR revealed that AUF1 binds c-myc mRNA in cells. Our results reveal a new paradigm for tumor metastasis and invasion in breast cancer.

15. SUBJECT TERMS
Breast cancer; RNA-binding proteins; AUF1; HuR; mRNA stability

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INTRODUCTION

The level of a messenger RNA depends not only on its rates of synthesis, processing, and transport, but also its rate of turnover. The turnover rate of an mRNA can, in turn, determine its lifetime as a template for protein synthesis. It is particularly important to understand how the levels of mRNAs encoding oncoproteins and cell cycle proteins are regulated because sustained synthesis of these gene products favors proliferation rather than differentiation, a hallmark of the neoplastic phenotype. Many cell cycle and proto-oncogene mRNAs exhibit extremely short half-lives. Their decay is controlled in part by A+U-rich elements (AREs) located in the 3'-untranslated region. Moreover, the half-lives of their mRNAs are frequently subject to regulatory control. This control is exerted via a balance between the action of at least two ARE-binding proteins, AUF1 and HuR. AUF1 targets the degradation of ARE-mRNAs such as the c-*myc* proto-oncogene and the cell cycle regulator cyclin D1. By contrast, HuR promotes stabilization of ARE-mRNAs. c-*myc* and cyclin D1 are of particular importance, since both play causative roles in mammary tumorigenesis. Our central hypothesis is that AUF1 may act as a novel tumor suppressor by limiting expression of genes that promote cellular proliferation. On the other hand, HuR may act as a novel oncoprotein by stabilizing those mRNAs. To address this hypothesis, we proposed to alter the expression of AUF1 or HuR in human breast carcinoma cells and examine the resulting effects on proliferation and tumorigenesis in a nude mouse model. There were two proposed phases to this work: (I) to examine the effects of AUF1 and HuR expression levels on global gene expression in cultured cells; and (II) to assess the roles of AUF1 and HuR in proliferation and tumorigenesis *in vivo*. For Phase I we have successfully introduced into human breast carcinoma cells an expression vector for knockdown of AUF1. Overexpression of AUF1 has proven difficult, as has stable knockdown of HuR. However, knockdown of AUF1 elevates expression of c-*myc* at the protein level, but not at the mRNA level, to our surprise. For Phase II, we have begun characterizing clones for their growth phenotypes with the goal of examining their ability to induce tumors as xenographs on nude mice. We have made an exciting discovery – knockdown of AUF1 appears to accelerate proliferation of breast carcinoma cells and ablate their cell-cell adhesion. Our prediction is that these cells will be viscously metastatic in xenograph assays. These findings introduce a new paradigm by which loss of an RNA-binding protein, AUF1, may lead to formation of highly aggressive, metastatic tumors.

BODY

The approved SOW is as follows:

Task 1. To examine the effects of AUF1 and HuR expression levels on gene expression in cultured breast carcinoma cells (Months 1-24):

a. Construct Tet-Off plasmids for overexpression of AUF1 and HuR (Months 1-4).
b. Construct plasmids for RNA interference (RNAi)-based knockdown of AUF1 and HuR (Months 1-4).
c. Transfect plasmids into breast carcinoma Tet-Off cells and select individual clones (Months 4-24).
d. Characterize clones for AUF1 and HuR expression levels to define those with low, medium, and high levels of AUF1/HuR expression (Months 6-24).
e. Using RNA from engineered cell lines, perform DNA microarray analyses of genes affected by altered AUF1/HuR expression, in particular, those involved in the cell cycle, invasion, metastasis, and angiogenesis (Months 6-24).
f. Identify those transcripts that are direct binding targets of AUF1 and HuR (Months 6-24).

Task 2. To assess the roles of AUF1/HuR in cell growth and tumorigenesis (Months 6-36):

a. Characterize the growth phenotypes of engineered cell lines obtained from Task 1d (Months 6-24).
b. Characterize the cell cycle distribution of these cell lines by flow cytometry (Months 6-24).
c. Introduce clones onto nude mice and score tumor formation as a function of AUF1/HuR expression levels and growth/cell cycle phenotypes (Months 6-36).

Since this is a final progress report, I will summarize work conducted over the entire research period. For Tasks 1-a and -c, we completed preparation of tetracycline-regulated expression constructs for $p37^{AUF1}$ (plasmid pTRE/p37), $p40^{AUF1}$ (plasmid pTREaI/p40), and HuR (plasmid pTREaI/HuR). Plasmids were transfected into MCF-7 Tet Off cells (human breast carcinoma) and drug selection yielded clones for each construct. Individual clones were picked from tissue culture plates and expanded to the point that they could be frozen in liquid nitrogen. At the same time, whole cell lysates were prepared from these clones for western blot analyses. We performed western blot analyses of the lysates to score for AUF1 or HuR overexpression (Task 1-d). Unfortunately, we have been unable to overexpress $p37^{AUF1}$, $p40^{AUF1}$, or HuR. We speculate that MCF-7 cells may possess mechanisms to maintain these proteins at specific levels. This is not so unusual; for example, cells do not permit overexpression of histones, the proteins that package DNA into chromosomes.

Figure 1. See text for details.

Nonetheless, for Tasks 1-b, -c, and -d, we completed preparation of a tetracycline-regulated expression construct encoding a short hairpin RNA (shRNA) designed to knockdown expression of all four AUF1 isoforms (Figure 2A, next page) by RNA interference (RNAi). As a prelude to transfecting this vector, named pS U6/tetO/hyg/AUF1/652, into MCF-7 Tet Off cells and selecting clones, we performed transient transfections with this plasmid to insure that the shRNA would indeed knockdown protein expression. Figure 1 is a western blot of such a pilot experiment with the AUF1 shRNA vector.

Clearly, this shRNA is very effective for RNAi-induced knockdown of AUF1 [Figure 1, compare lane 2 (AUF1 shRNA) to lane 1 (control)]. We then proceeded with transfections for selection of clones. We also constructed a vector encoding a shRNA with random sequence to serve as a control (referred to as shCTL). Clones were selected, expanded, and frozen in liquid nitrogen. We froze two clones for the control shRNA (shCTL) and nine clones for the AUF1 shRNA. Lysates were prepared for western blot analyses of AUF1 levels using cells expressing the control shRNA (shCRL) and clones expressing the shRNA directed against AUF1. Our intent was for the shRNAs to be inducible by culturing cells in doxycycline (Dox). However, as Figure 2B demonstrates, AUF1 knockdown occurs even in the absence of Dox for all clones tested. Note that AUF1 is readily detected in cells expressing the control shRNA, labeled as shCTL. We estimate that AUF1 knockdown is at least 95% in all nine clones tested.

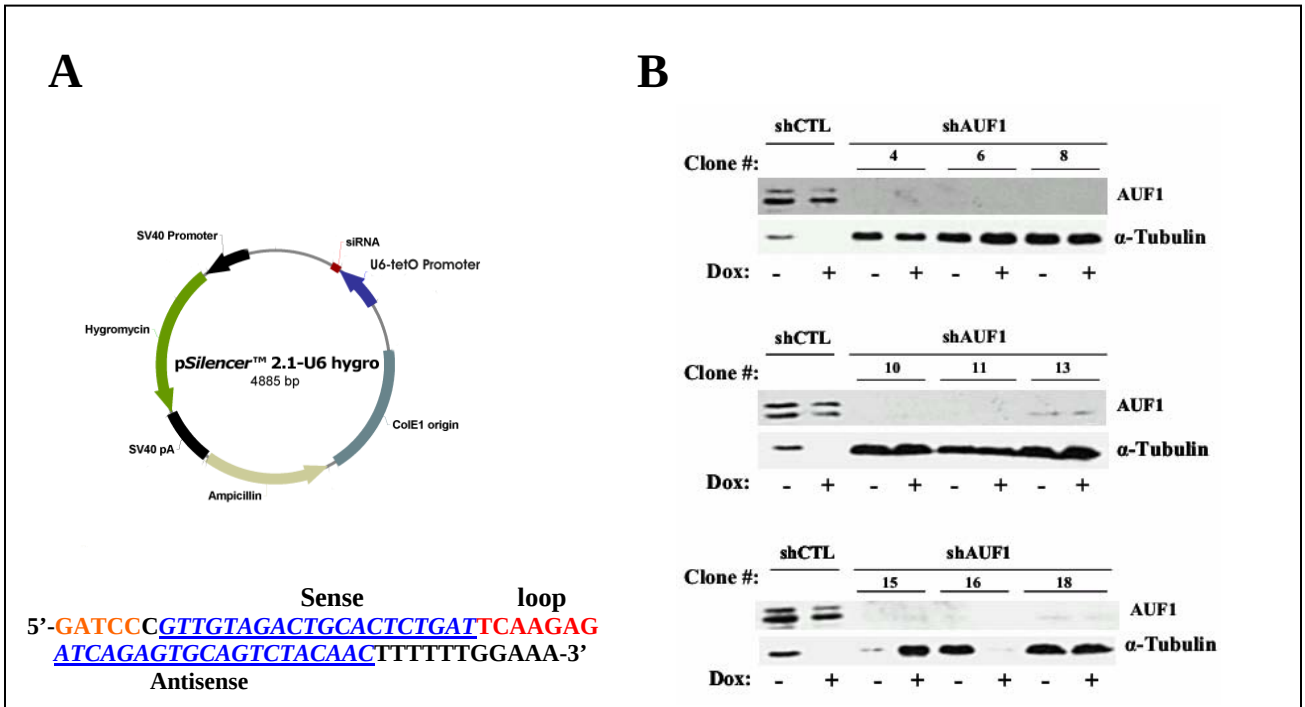

Figure 2. Vector construction for knockdown of AUF1 and creation of stably transfected cell lines. A) pSilencer U6-tetO vector map (modified from www.ambion.com). Short hairpin RNA against AUF1 is transcribed from pSilencer. Below map, complete short hairpin sequence cloned into the modified pSilencer. B) MCF-7 cells were stably transfected with pS U6/tetO/hyg/AUF1/652, drug selected, and expanded. A number of cells were harvested for each clone and fractionated by SDS-PAGE, followed by western blot analyses for AUF1 and α-Tubulin.

We experienced problems finding a shRNA for HuR knockdown. Fortunately, Pan et al. (J. Biol. Chem. 280: 34609, 2005) described a vector that permits 70-80% knockdown of HuR. We obtained their vector, transfected it into MCF-7 Tet Off cells, and attempted to select clones (Task 1-d). However, we were unable to obtain clones with HuR knockdown. We speculate that long term knockdown of HuR is lethal. In retrospect, this is not surprising, since HuR serves to maintain levels of important growth regulatory mRNAs/proteins – without HuR, these genes are effectively silenced. Tumorigenesis experiments, however, would require long-term knockdown of HuR. We have thus focused our attention exclusively on the effects of AUF1 knockdown on breast carcinoma cells.

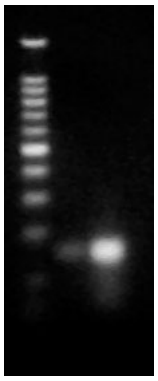

Figure 3. See text for details.

As a prelude to identifying the mRNA target subset for AUF1 in MCF-7 cells (Task 1-f), we established the conditions for mRNP immunoprecipitation (RIP) assays. This assay utilizes an antibody to an RNA-binding protein to immunoprecipitate mRNPs associated with that protein. We immunoprecipitated mRNPs with our AUF1 antibody or preimmune serum (as a control) and purified the RNAs in the precipitates. To verify that we purified AUF1 target mRNAs, we performed quantitative RT-PCR (qPCR) using primers and a fluorescent probe specific for c-*myc* mRNA, a suspected AUF1 target. The C_t with the preimmune sample was 32.54 and the C_t for the AUF1 antibody sample was 29.24. The ΔC_t represents a ~8-fold difference and demonstrated that the AUF1 antibody immunopurified c-*myc* mRNA. The products of the qPCR reaction were fractionated in an agarose gel to verify that the PCR product was the predicted size. Lane 1 in Figure 3 contains 100-bp increment size markers. (The brightest is 500 bp.) The gel indicates that the PCR products in Figure 3, lanes 2 and 3, are ~150 bp, the predicted size. Note also that the intensity in lane 3 (the anti-AUF1 sample) is much higher than lane 2 (the control sample), consistent with the qPCR results. We have experienced problems with our in-house cDNA Microarray Facility. Consequently, we have been greatly delayed in identification of global AUF1 target mRNAs from RIP assays. Thus, we are preparing to perform the microarray experiments ourselves.

Figure 4. See text for details.

Nonetheless, the results of Figure 3 demonstrate that c-*myc* mRNA is a target of AUF1. We then decided to examine the effects of AUF1 knockdown on c-*myc* expression. For pilot experiments, we chose AUF1-knockdown clone 13 (see Figure 2). Lysates of cells expressing either the control shRNA (clone D2) or clone 13 were analyzed by western blot for expression of AUF1, c-Myc, and α-Tubulin (the internal control). Figure 4 again demonstrates AUF1 knockdown. Interestingly, c-Myc levels were elevated almost 4-fold after normalization to α-Tubulin. However, qPCR indicated that AUF1 knockdown does not alter c-*myc* mRNA levels. This indicates that AUF1 acts to repress translation of c-*myc* mRNA, an unexpected, but highly significant result. We are now working to define the molecular mechanisms of AUF1-dependent c-*myc* regulation.

In regard to Task 2, we began characterizing the growth phenotypes of clones expressing either the control shRNA or the AUF1 shRNA. We initially compared growth curves for control clone D2 and AUF1 shRNA clone 13. As shown in Figure 5 (next page), knockdown of AUF1 has an impressive effect on proliferation rate, i.e., it's significantly faster than the control cells. This is not too surprising, given that AUF1 knockdown elevates c-Myc levels. It has been known for decades that c-Myc expression promotes cell proliferation. However, to be thorough, we are in the process of examining additional clones to ascertain that the effects are not due to an artifact of integration site of the shRNA vector within a chromosome.

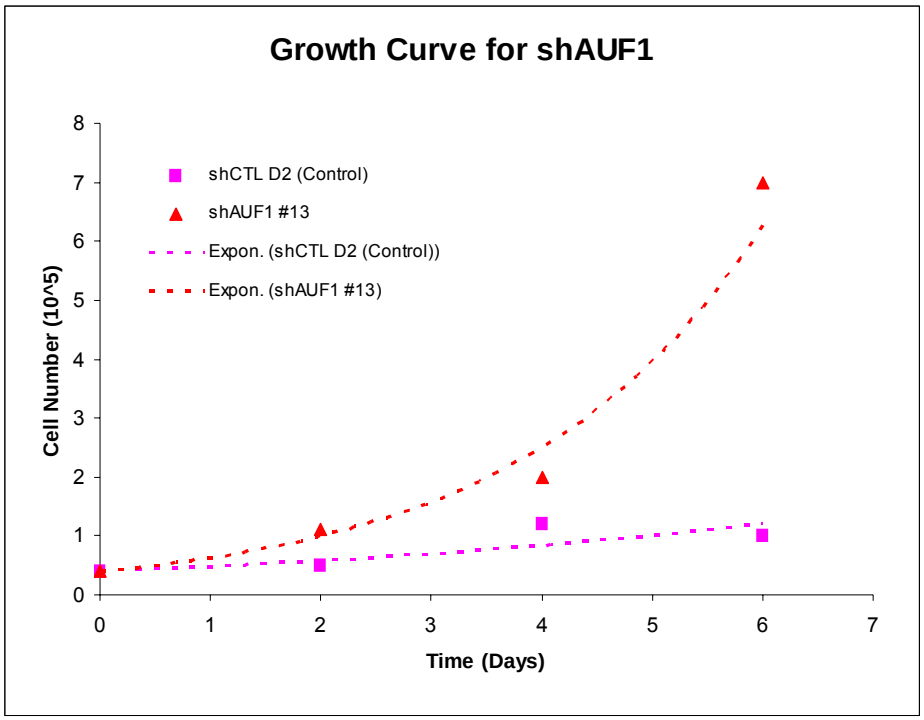

Figure 5. Growth curves for MCF-7 cells transfected with the control shRNA (shCTL) or shRNA directed against AUF1.

What is undoubtedly the most exciting observation we have made is that MCF-7 cells with AUF1 knockdown are altered in their cell-cell adhesion properties. Normally, MCF-7 cells are quite difficult to disperse, even after trypsin treatment. By contrast, cells with knockdown of AUF1 are readily dispersed – even in cell culture, they do not form the epithelial "sheets" that control cells do. Importantly, all clones with AUF1 knockdown exhibit a similar defect in cell-cell adhesion. We are now planning xenograph experiments with nude mice. Our prediction is that AUF1 knockdown cells will possess a vicious, metastatic phenotype. The potential implications of this result are immense – highly metastatic breast cancer in some patients may result in part from inactivation or loss of AUF1 expression. In future studies beyond the scope of this grant, we will examine primary breast tumors from patients to look for such loss of AUF1 expression as a predictor of metastasis.

Finally, we have succeeded in constructing a truly genetic system to explore AUF1's effects on proliferation and cell adhesion in breast carcinoma cells. Briefly, we mutated the shRNA binding site within the AUF1 cDNAs for each of the four isoforms so that the shRNA can no longer trigger RNAi for that mRNA (Figure 6A, next page). We use the designation $AUF1^{R}$ for the cDNAs encoding the shRNA-**R**esistant mRNAs. We are currently cloning these into the pTRE vector so that we can induce their expression by withdrawal of Dox from either growth medium (for cell culture studies) or drinking water (for animal studies). To demonstrate that this complementation strategy will work, we transfected either the wild-type $p40^{AUF1}$ or $p40AUF1^{R}$ into MCF-7 clone 13 and examined $p40^{AUF1}$ protein levels by western blot. Figure 6B shows that expression of $p40^{AUF1}$ is restored upon transfection of $p40AUF1^{R}$; transfection of wild-type $p40^{AUF1}$ permits only minimal expression, as expected. Thus, we can complement cells with any AUF1 isoform/s in a background where all other isoforms are absent. For animal studies, we plan to implant cells transfected with $AUF1^{R}$ vectors into nude mice and either turn-on or turn-off expression of the selected AUF1 isoform to examine tumor proliferation and metastasis. We will even express isoforms in varying

combinations. We will then perform microarray analyses of cells complemented with a selected isoform. This strategy will permit us to assign functions to individual AUF1 isoforms, both in terms of tumorigenesis/metastasis as well as gene expression. The power of this genetic system for breast cancer research can not be overemphasized!

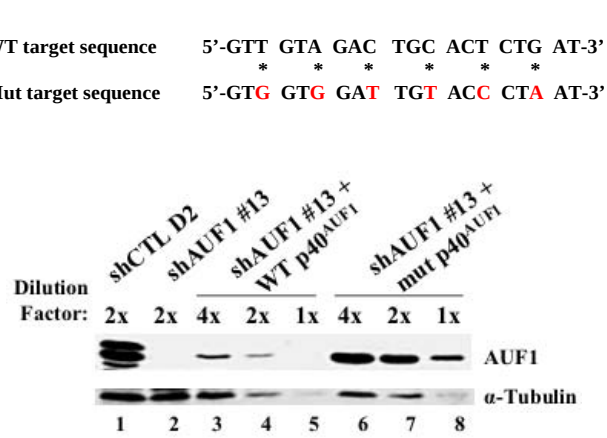

Figure 6. Restoration of AUF1 protein levels in clone 13. A) Mutations were made in the AUF1 coding region in order to create resistance to the short hairpin in knockdown clones. These mutations permit restoration of $p40^{AUF1}$ but do not change the amino acid sequence. B) Transient transfection of wild-type $p40^{AUF1}$ (lanes 3, 4 and 5) and $p40AUF1^{R}$ (lanes 6, 7 and 8) into AUF1 knockdown clone 13. Lane 1 shows AUF1 protein levels in a control cell line stably transfected with a scrambled short hairpin sequence. Lane 2 shows AUF1protein levels in clone 13 prior to transient restoration of $p40^{AUF1}$.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of vectors for overexpression or knockdown of AUF1 and HuR.
- Creation of cloned cell lines of MCF-7 with >95% knockdown of AUF1 expression compared to cell lines expressing a control shRNA.
- Identification of c-*myc* mRNA as an AUF1 target mRNA in cells.
- Discovered that AUF1 is a novel translational repressor of c-*myc* mRNA.
- Knockdown of AUF1 accelerates proliferation of MCF-7 cells and disrupts their cell-cell adhesion properties.
- Designed a genetic system for complementation of individual AUF1 isoforms in knockdown cells to assess the effects on cell proliferation and cell-cell adhesion in culture and in animals.

REPORTABLE OUTCOMES

We have built the tools that will allow us to assess the biological effects of reengineering expression of the key ARE-binding protein AUF1 in human breast carcinoma cells. We have also made two seminal observations: AUF1 is a translational repressor of c-*myc* mRNA; and AUF1 knockdown blocks the cell's ability to form stable cell-cell contacts. These observations, as such, are not yet reportable outcomes. However, each observation serves as a foundation for publishable studies. We thus envision at least two, very high impact publications emanating from the work described here: one detailing the molecular mechanisms of c-*myc* translational regulation by AUF1 and the other describing molecular mechanisms by which AUF1 knockdown alters the tumor biology of MCF-7 cells.

CONCLUSIONS

Our central hypothesis has been that AUF1 may act as a novel tumor suppressor by limiting expression of genes that promote cell growth, and that HuR may act as a novel oncoprotein by stabilizing those mRNAs. Our approach was to alter the expression of AUF1 or HuR in human breast carcinoma cells and examine the resulting effects on cell growth and tumorigenesis in a nude mouse model. Our work had two phases: (I) to examine the effects of AUF1 and HuR expression levels on gene expression in cultured cells; and (II) to assess the roles of AUF1 and HuR in cellular proliferation and tumorigenesis *in vivo*. We completed expression vector constructions, transfections, and clone selections germane to Phase I. Overexpression of either AUF1 or HuR was problematic, as was knockdown of HuR. Knockdown of AUF1 was achieved, however, and led to two very important, seminal observations: (i) AUF1 acts to repress translation of c-*myc* mRNA to limit protein synthesis. As such, AUF1 does operate as a tumor suppressor. (ii) Knockdown of AUF1 disrupts the cell-cell adhesive properties of breast epithelial cells. We predict that these cells will exhibit a highly aggressive, metastatic phenotype. We developed a means to complement expression of any AUF1 isoform within a background of knocked down, endogenous AUF1 expression. This novel and powerful genetic system will be a gold mine for dissecting new mechanisms and pathways by which breast carcinoma cells become highly metastatic during advanced breast cancer. The three years we have devoted to this project are only the beginning. I predict that this work will ultimately reshape our thinking about the many roads that lead to tumorigenesis. Along the way, our work will surely lead to new therapeutic paradigms as well.

REFERENCES

None

APPENDICES

None

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PERSONNEL RECEIVING PAY

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