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TITLE: **RNA Binding Proteins Posttranscriptionally Regulate Genes Involved in Oncogenesis**

PRINCIPAL INVESTIGATOR: **Ulus Atasoy, MD., MA, J. Wade Davis, PhD., Tim Hoffman, PhD.**

CONTRACTING ORGANIZATION: **University of Missouri, Columbia, MO. 65212**

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14. ABSTRACT

The purpose of the proposed research is to better understand posttranscripti onal gene regulation in breast cancer. **Our hypothesis is that the RNA binding protein Hu R increases cell proliferatio n by regulating mRNAs involved in breast oncogenesis at the posttr anscriptional leve l.** HuR has been described as regulating at the posttranscriptional level, genes involved in malignant transformation. We have optim ized techniques of **R**NA **i**mmuno**p**recipitations (RIP-Chip) from both ER+ and ER- cell lines, perform ed genome wide array analysis and have identified distinct subsets of HuR associated m RNAs in different breas t can cer cell lines. Additionally, we have also made stably transfected breast cancer cell li nes which over-express and under-express HuR. As predicted by our hypothesis, cells which over expr ess HuR grow m ore aggressively. Ho wever, unexpectedly, when ER - cells which over-expressed HuR were injecting into athym ic nude m ice, there was a si gnificant reduction in tum or growth, as compared to control tumors. The putative m echanisms seem to be anti-angiogenic effect upon key genes involved in angiogenesis.

15. SUBJECT TERMS
RNA binding proteins; estrogen receptor negative breast cancer, posttranscriptional gene regulation, RNA immunoprecipitation applied to microarrays (aka RIP-Chip)

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Table of Contents

INTRODUCTION

Whereas transcriptional gene regulation is well studied, posttranscriptional gene control is poorly understood. Yet, emerging evidence indicates that posttranscriptional control by RNA binding proteins (RBPs) and microRNAs (miRNAs) are important key regulators of gene expression of many cancer related genes. The RBP, HuR, is a master regulator of many early response genes in cancer. Of the six classical acquired traits first proposed by Hanahan and Weinberg that malignantly transformed cells develop, HuR has been demonstrated to control genes in multiple areas.

The purpose of this research is to define the *in vivo* mRNA targets of the RNA binding protein, HuR using **R**NA **i**mmuno**p**recipitations applied to microarray chips (**RIP-Chip**) in estrogen positive (ER+) and estrogen negative (ER-) breast cancer. Additionally, we will test the **hypothesis that HuR increases cell proliferation by regulating mRNAs involved in breast oncogenesis at the posttranscriptional level.** We will do this by altering HuR levels in breast cancer cell lines using lentiviral short hairpin RNAi to knock down and also over-express HuR and assay tumor formation by injection into athymic nude mice (orthotopic model).

BODY OF WORK

The funding from the DOD resulted in two publications, which are listed in the Appendix and References: Calaluce et al and Gubin et al. In the first publication, Calaluce et al, we described how we developed methods of RIP-Chip and applied them to both ER- and ER+ breast cancers, MDA-MB-231 and MCF-7, respectively. In **Figure 1**, (Calaluce et al) we see that HuR RIP is able to identify a known HuR target, β-actin mRNA robustly in both cell types. We then extended these RIP-Chip techniques to genome wide arrays. In **Figure 2** (Calaluce et al), re-produced below, we see a statistical compilation of three independent immunoprecipitations with matching controls in both cell lines. There are three classes of HuR associated mRNAs: those associated with HuR only in MDA-MB-231 cells (upper left), those only with MCF-7 (lower right) and mRNAs that are HuR associated with both cells types.

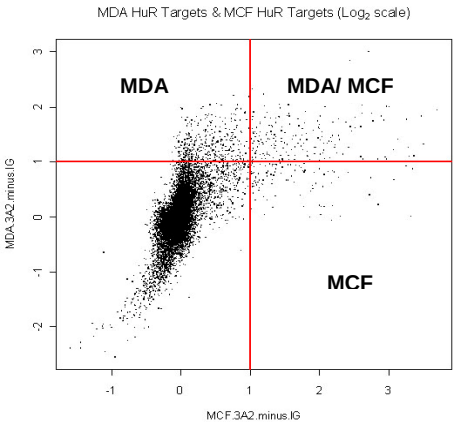


Figure 2: HuR pull downs reveal unique sets of mRNA targets in breast cancer cell lines. HuR pull downs were performed from MCF-7 or MDA MD 231 lysates using HuR antibody (3A2) and IgG1 isotype control and hybridized to Illumina Sentrix arrays (47,000 genes). Control signals are subtracted. Above results represent cumulative data from 12 different arrays. Experiments were done n=3. Scales are log2.(From Calaluce et al.)

We performed Gene Ontology (GO) classification of these genes are suggested putative novel members to the acquired capabilities model of cancer as proposed by Hanahan and Weinberg (**Figure 3** , Calaluce et al.). The original RIP-Chip results were confirmed using RT-PCR for two target genes, calmodulin 2 (*CALM2*) and *CD9* (**Figure 4** , Calaluce et al). These genes were chosen since they have known cancer associations and were highly represented as HuR target mRNA in both cell types. As seen in Figure 4, HuR immunoprecipitation efficiently recovers an enrichment of both *CALM2* and *CD9* as compared to isotype controls.

We verified the RIP-Chip results using a second independent method, biotin pull downs. In biotin pull downs, the relevant portion of the target mRNA is in vitro transcribed with biotin-UTP and this extracted mRNA is then mixed with cellular lysate which contains HuR protein. The complex is recovered and then analyzed by Western. As seen in the results in **Figure 5** (Calaluce et al), both *CALM2* and *CD9* have predicted HuR binding sites in the 3' UTR and biotin pull downs indicate that HuR interacts with only the 3' UTR but not any other region of the mRNAs. We conclude that by two independent methods, RIP-Chip and biotin pull downs, HuR is indeed physically associating with *CALM2* and *CD9* mRNAs.

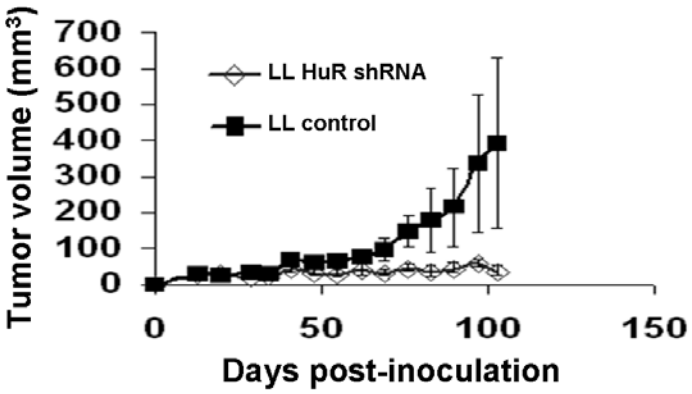
Physical association between HuR and mRNA targets does not, however, prove functionality. In order to test whether HuR is controlling expression of these two target mRNAs, we made mutant cell lines, MDA-MB-231 and MCF-7 which over-expressed or under-expressed HuR. As seen in **Figures 6 and 7** (Calaluce et al), using lentiviral vectors which express shRNA HuR, we were able to knock-down HuR by over 90% in both cell lines. We also developed stably transfected clones which over-expressed epitope (HA) tagged HuR by different amounts in both MDA-MB-231 and MCF-7 cells. However, unexpectedly, over-expression of HuR in MB-231 cells resulted in *decreases* in both *CD9* mRNA and protein (**Figure 6** , Calaluce et al) but the levels of *CALM2* were not decreased but rather *increased*. However, the results in MCF-7 cells were as predicted: HuR over-expression resulted in increased in both CD9 and CALM2 mRNA and protein (**Figure 7** , Calaluce et al). Therefore, we conclude that altering HuR levels affect the same genes differently, depending upon cellular milieu.

We believe that the importance of this work lies in two areas: firstly, we were able to identify cancer-associated genes as novel HuR targets, which was previously unknown. Secondly, we believe that the RIP-Chip methods may have uncovered novel cancer genes. There were many genes which were strongly recruited to the HuR pellet in both cell types, which do not have any known cancer functions.

Since HuR generally affects genes by increasing their expression, these may well represent genes which are recruited to the polysomes to make more protein.

To more fully investigate the functional consequences of altering HuR expression in ER- breast cancer, we injected MDA-MB-231 cells with increased levels of HA-HuR expression in athymic nude mice. The results are described in the second publication, Gubin et al. In **Figure 1** (Gubin et al), we see that MB-231 cells which over-express HA-HuR grow significantly faster *in vitro* with alterations in cell cycle kinetics. Unexpectedly, when they were introduced orthotopically into mammary fat pads of female athymic nude mice, they produced much smaller tumors as compared to empty vector (EV) controls (**Figure 1D ,** Gubin et al). This surprisingly result was replicated with a second experiment, which included parental MDA-MB-231 cells (**Figure 2A**, Gubin et al). There is a 90% reduction both in tumor size and mass of HA-HuR tumors. Tumors dissected from animals contained live cells which are undifferentiated adenocarcinoma (**Figure 2D**). We performed gene profiling and identified genes which were up-regulated in HA-HuR tumors (**Table 1**, Gubin et al.). There were many genes of interest and we focused on known HuR target genes, such as thrombospondin1 (TSP1), and VEGF. Additionally , TSP2 was also identified, though this has not been appreciated to be a HuR target. We asked whether there are alterations of TSP1 and VEGF expression in HA-HuR tumors. There are increases in TSP1 expression at both the mRNA and protein levels, but decreases in VEGF (**Figure 3**, Gubin et al.). We verified that both TSP1 and VEGF mRNA interact with HuR in HA-HuR tumors and demonstrated that HuR over-expression results in increased stabilization of TSP1 but not VEGF mRNA half-life (**Figure 4**, Gubin et al.). These results are significant because TSP1 is known to be a potent inhibitor of angiogenesis, whereas, VEGF, is a potent pro-angiogenic gene. We ruled out putative explanations, such as alterations in senescence, proliferation or apoptosis. The putative mechanism seems to be a drastic reduction (75%) in blood vessels in HA-HuR tumors. We conclude that HuR over-expression does indeed result in much smaller tumors which has significant reductions in blood vessels.

Finally, we have used lentiviral vectors to **decrease** HuR levels in MDA-MB-231 cells by making stably infected clones (see **Preliminary Data Figure 1**). When these cells were injected into athymic nude mice, there were growth reductions of 95% in tumors with lower HuR levels, as compared to lentiviral vector controls. These surprising results are confusing and seemingly contradict our published results in Gubin et al. However, there is confusion in the literature whether HuR is a tumor suppressor or oncogene (see below).



Preliminary Data Figure 1: MDA-MB-231 cells infected with a lentivirus expressing a shRNA knocking down HuR (LL HuR shRNA) showed significantly reduced tumor volume (mm^3) and growth starting at seven weeks post-inoculation and continuing for fourteen weeks when compared to MDA-MB-231 infected with control lentivirus (LL control). Five animals per group were used; $p<0.05$.

RECOMMENED CHANGES FOR FUTURE WORK

Our findings for this application must be tempered by two salient facts: (1) athymic nude mice lack a normal immune system, which human patients possess; (2) we worked with only one type of ER- breast cancer, MDA-MB-231. Therefore, presently, it is not clear whether our findings can be extrapolated to other breast cancer types. The initial confusion in results, suggesting that over-expression and under-expression of HuR can both reduce tumor growth in animal models, is reflected in the literature over whether HuR is a tumor suppressor or oncogenes. Heinonen and colleagues demonstrated a direct correlation between increased cytoplasmic HuR and worse prognosis in breast cancer (Heinonen et al.). Ortega et al. arrived at the opposite conclusion (Ortega et al). Therefore, we conclude that further study is warranted to better understand the role of HuR in breast cancer.

For future work, we would suggest two broad approaches. First, we would suggest using a spontaneously occurring mouse breast cancer model, such as 4T1 Balb/c tumors in syngeneic animals. Syngeneic animals have normal immune systems. One could make 4T1 clones which over-express or under-express HuR and inject orthotopically into Balb/c mice and measure tumor growth and metastasis. Secondly, we have made a conditional HuR knock-out mouse. This mouse could be crossed with a genetically engineered mouse model of breast cancer, for example, MMTV-PyMT. Such a model would enable us to genetically ablate HuR and ask the questions whether this interferes with breast cancer growth and/or metastasis.

PERSONAL WHO RECEIVED SUPPORT

Dr. Ulus Atasoy (PI)
Dr. Wade Davis (Co-Investigator)
Dr. Tim Hoffman (Subcontract)
Mr. Matt Gubin (Graduate student)

KEY RESEARCH ACCOMPLISHMENTS

Outline of Statement of Work (SOW) (underlined and in red tasks have been completed)

Task 1

Specific Aim 1: Determine HuR targets in ER+ and ER- cell lines using RNAi and immunoprecipitation.

Experimental Design:

(1) perform transcriptomic and ribonomic analyses on ER+ and ER- cell lines
(2) make stable ER + and ER- cell lines which *under* express HuR using lentiviral RNAi
(3) form intersection (Venn diagram) of above two approaches to obtain HuR targets in ER+ and ER- cell lines
(4) validate HuR targets using Q-PCR

Task 2

Specific Aim 2: Measure growth characteristics of ER+ and ER- cell lines with altered HuR levels *ex vivo* and inject into nude mice.

Experimental Design:

(1) make stable ER+ and ER- negative cell lines which *over* express epitope tagged HuR
(2) measure growth characteristics of altered cell lines *in vitro*
(3) inject modified ER+ and ER- cell lines into athymic nude mice and monitor tumor growth
(4) remove tumors from test animals, perform transcriptomic and ribonomic analyses to measure HuR targets using Q-PCR, Western blotting and immunohistochemical staining

Task 3

Specific Aim 3: Determine whether HuR knockdown by RNAi shrinks established tumors in nude mice.

Experimental Design:

(1) Establish ER+ and ER- tumors in nude mice
(2) Inject lentivirus expressing HuR RNAi, measure tumor growth
(3) Remove tumors and perform transcriptomic and ribonomic analyses to determine effects upon HuR and top ten targets using Q-PCR, Western and immunohistochemical staining

- Identification of distinct sets of HuR associated mRNAs from ER- and ER+ breast cancer lines
- Development of stably transfected ER+ and ER- breast cancer cell lines with increased and decreased HuR levels
- Development of lentiviral vectors which over-express and under-express HuR
- Identification and validation of novel HuR associated mRNAs from ER- and ER+ breast cancer (*cd9* and *calm2*)
- Identification of novel cancer related genes which are HuR targets
- HuR differentially regulates the same mRNA targets (*cd9* and *calm2*) depending upon cellular milieu
- Demonstration that changes in HuR levels in ER- breast cancer results in alterations in cell cycle kinetics and altered growth rates
- Demonstration that HuR **over-expression** in ER- breast cancer increases *in vitro* growth rates but in orthotopic models results in significant **decreases** (90 %) in tumor size by interfering with angiogenesis
- Demonstration that HuR **over-expression** in ER- breast cancer results in significant (75 %) reductions in angiogenesis

- **Knock-down** of HuR in ER- (MDA-MB-231 cells) using lentiviral shRNA vectors results in significant (95%) **reduction** in tumor size in orthotopic models of breast cancer

Work with ER+ cell lines (MCF-7) with altered HuR levels was hampered by their inability to grow *in vitro*. Despite several attempts at re-making MCF-7 cells with higher and reduced levels of HuR and derving stable clones, these cells did not appreciably grow *in vitro* and therefore we were unable to transplant into athymic nude mice.

We made lentiviral vectors which over-expressed and under-expressed HuR but did not complete the experiments outlined in Task 3 (" **Determine whether HuR knockdown by RNAi shrinks established tumors in nude mice"),** due to time constraints. Instead, we focused and followed up our surprising findings in Tasks 1 and 2 that HuR over-expression reduced tumor growth with a detailed investigation into mechanisms of tumor reduction. Our putative explanation is that HuR over-expression results in significant reductions in angiogenesis which interferes with tumor growth. The mechanism involves increases in TSP-1 (a potent angiogenic inhibitor) by mRNA stabilization and a concomitant decrease in VEGF (potent pro-angiogenic factor) expression.

As per instructions requesting complete research methodologies, we have included two manuscripts which have been accepted for publication based upon the data generated above; Calaluce et al and Gubin et al. these two manuscripts are included as attachments in the **Appendix**. These manuscripts describe in detail all the methodologies and research results in detail.

Milestones

Year 1	completed
Year 2	completed
Year 3	initiated
Task 1	completed
Task 2	mostly completed
Task 3	initiated, partially completed, not finished

REPORTABLE OUTCOMES

(1) Magee, Joe, Gubin, Matt, Atasoy, Ulus. Coordinate Posttranscriptional Regulation of Cancer Genes by the RNA Binding Protein HuR. *Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications (San Diego, October 17-20, 2007)*

(2) Magee, Joe, Gubin, Matt, Atasoy, Ulus. Coordinate Posttranscriptional Regulation of Cancer Genes by the RNA Binding Protein HuR. *AACR Annual Meeting (San Diego, April 12-16, 2008)*

(3) Magee, Joe, Gubin, Matt, Atasoy, Ulus. Coordinate Posttranscriptional Regulation of Cancer Genes by the RNA Binding Protein HuR. *Era of Hope CDRMP Meeting (Baltimore, June 25-28, 2008)*

(4) Magee, Joe, Gubin, Matt, Atas oy, Ulus. RNA Immunopr ecipitations (RIP-on-Ch ip) Identify Unique Subsets of Genes in ER+ and ER- Breast Cancer. *AACR Annual Meeting (Denver, April 18-22, 2009; oral presentation)*

(5) Calaluce, Robert, Gubin, Matt, Magee, Joe, Atasoy, Ulus. The RN A binding protein, HuR, regulates distinct subsets of m RNAs in estrogen receptor negative (ER-) and estroge n rec eptor pos itive (ER+) breast cancer. *AACR Special Symposium on Breast Cancer (San Diego, October 14-17, 2009; poster presentation)*

(6) Gubin, Matt, Calaluce, Robert, Ma gee, Joe, Casolaro, Vincenzo, Stellato, Cristiana. The RNA binding protein, HuR, coordinately re gulates mRNAs in breast cancer and C D4+ Th2 polarization. *Keystone Symposium (Keystone, CO, February 7-12, 2010; poster presentation).*

(7) Gubin, M, Calaluce, Bob, Davis, W ade, Magee, Joe, Strouse, Connie, Shaw, Daniel, Hoffm an, Tim, Rold, Tammy, Atasoy, Ulus. The RNA binding protein HuR controls a ngiogenesis in triple negative breast cancer. *AACR Annual Meeting (Washington, DC, April 15-21, 2010; poster presentation)*

(5) Robert Calaluce, Matthew M. Gubin, J. Wade Davis, Joseph D. Magee, Yuki Kuwano, Myriam Gorospe, Ulus Atasoy. The RNA binding protein, HuR, differentially regulates unique subsets of mRNAs in estrogen receptor negative and estrogen receptor positive breast cancer. *BMC Cancer 2010, 10:126*.

(6) Matthew M. Gubin, Robert Calaluce, J. W ade Davis, Joseph D. Magee, Connie S.Strouse, Daniel P. Shaw, Tim othy Hoffm an, Tammy L. Rold, Ulus Atasoy. Over-expres sion of the RNA binding protein HuR im pairs tum or growth in triple negative breast cancer by interferin g with angiogenesis. *Cell Cycle 9:16, 1-11; August 15, 2010.*

CONCLUSIONS

We have performed RIP-Chip (RNA immunprecipitation applied to microarrays, (chips)) analysis from ER- and ER+ breast cancer cell lines and have identified unique, distinct HuR signature subsets of mRNAs and validated novel HuR target genes. HuR differentially regulates the same mRNA targets in different fashion depending upon cellular milieu. These methods may be used to identify novel breast cancer targets, missed by traditional gene profiling methods.

Using orthotopic models of breast cancer in athymic nude mice, we have made the finding that HuR overexpression accelerates MDA-MB-231 (ER-) breast cancer growth *in vitro*, but surprisingly, results in a significant (90%) reduction in tumor growth as compared with controls by interfering with angiogenesis. The putative mechanisms seem to be an anti-angiogenic effect in that HuR over expression increases the expression of thrombospondin 1 (TSP-1) a potent inhibitor of blood vessel formation and simultaneously decreases VEGF. In parallel, we have also used lentiviral shRNA to knock-down HuR in ER- breast cancer; when injected into athymic nude mice; this also resulted in significant reduction in tumor growth.

"So what": These methods will enable us to clarify the role of HuR in breast cancer as well as the identification of novel HuR regulated mRNA targets which may play roles in breast cancer. Additionally, these results are highly significant because if validated, HuR may be controlling a posttranscriptional operon which regulates angiogenesis in ER- tumor formation. Thus, these findings may establish a new paradigm of treatment in aggressive triple negative breast cancer for which currently mono-therapies do not exist. Additionally, we have developed robust lentiviral vectors which can over-express and under-express HuR. These tools can be used to infect a variety of cell types.

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2. Gubin, M. M., R. Calaluce, J. W. Davis, J. D. Magee, C. S. Strouse, D. P. Shaw, L. Ma, A. Brown, T. Hoffman, T. L. Rold, and U. Atasoy. 2010. Overexpression of the RNA binding protein HuR impairs tumor growth in triple negative breast cancer associated with deficient angiogenesis. *Cell Cycle* 9:3337-3346.

3. Heinonen, M., P. Bono, K. Narko, S. H. Chang, J. Lundin, H. Joensuu, H. Furneaux, T. Hla, C. Haglund, and A. Ristimaki. 2005. Cytoplasmic HuR expression is a prognostic factor in invasive ductal breast carcinoma. *Cancer Res* 65:2157-2161.

4. Ortega, A. D., S. Sala, E. Espinosa, M. Gonzalez-Baron, and J. M. Cuezva. 2008. HuR and the bioenergetic signature of breast cancer: a low tumor expression of the RNA-binding protein predicts a higher risk of disease recurrence. *Carcinogenesis* 29:2053-2061.

APPENDIX

(1) See attached two manuscripts, Calaluce et al (*BMC Cancer 2010, 10:126*) and Gubin et al. (*Cell Cycle 9:16, 1-11; August 15, 2010*).

http://www.ncbi.nlm.nih.gov/pubmed/20370918

http://www.ncbi.nlm.nih.gov/pubmed/20724828

Please see end of Appendix for original PDF versions of these two publications.

(2) Magee, Joe, Gubin, Matt, Atasoy, Ulus. Coordinate Posttranscriptional Regulation of Cancer Genes by the RNA Binding Protein HuR. *Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications (San Diego, October 17-20, 2007)*

Coordinate Posttranscriptional Regulation of Cancer Genes by the RNA Binding Protein HuR
Magee, Joe, Gubin, Matt, Atasoy, Ulus. University of Missouri, Columbia, Missouri.

Due to the poor correlation betw een steady stat e m RNA levels and protein products, trad itional microarray analysis m ay m iss m any genes which are regu lated primarily at the level of mRNA stability and translation. Posttranscriptional gene regulation is becoming increasi ngly recognized as an important form of cellular control. W hereas our understanding of transc riptional regulation is advanced, in contrast this area rem ains largely unexplored. Yet, increa singly, the im portance of microRNAs and RNA binding proteins (RBPs) is now beginning to be better appreciated. We study the *elav* (embryonic lethal abnormal vision) fam ily of RBPs, which are paraneoplastic antigens, over-express ed in a variety of m alignancies, including breast cancer. Antibodies against elav family members are believed to be cancer-protective. The *elav* family of RBPs binds to the AU-rich elements (AREs) found in the 3' unt ranslated regions (UTRs) of many early-response genes, including proto-oncogenes and cell cycle regulators. HuR, the ubiquitously expressed family member, has recently been described to play a role in cancer progression. HuR stabilizes and translationally up regulates the expression of its target mRNAs. El evated levels of cytoplasm ic HuR directly correlate with increased invasiveness of m alignancy and poor prognos is for m any cancers, including those of the breast. HuR has been descri bed to control the expres sion of genes in all six different areas of transform ation originally descri bed by Hanahan and W einberg. Hence, it has been recently suggested that HuR may serve as a tumor m aintenance gene which allo ws for cancers, once they are established, to proliferate. Therefore, it is of interest to discover in vivo Hu R targets, a s these genes may play vital roles in transform ed cells. W e have developed m ethods which enable us to identify en masse, in vivo targets of RBPs such as HuR fr om cell lines and now for the f irst time, solid tissues as well. W e call this ribonom ic analysis, in which we i mmunoprecipitate subset s of ribonuclear particles (RNPs), exact the m RNA and then apply to m icroarrays. T his technique, in which no cross linking is used, allows one to en ma sse identify different in vivo m RNA targets which different RBPs interact with. Using a combination of HuR i mmunoprecipitation and RNA interference, we have been able to identify HuR in viv o targ ets which m ay play im portant ro les in tu mor m aintenance. Fur thermore, we pres ent evidence of cross talk am ongst st abilizer and de-stabilizer RBPs wh ich can potentially regulate the expression of multiple cancer targets. These techniques have the potential to better delineate gene s whose steady state mRNA levels m ay not significantly change, but which are tr anslationally active inside cancer cells. Potentially the identification of such genes m ay offer novel therapeutic targets to inhibit cancer growth.

(3) Magee, Joe, Gubin, Matt, Atasoy, Ulus. Coordinate Posttranscriptional Regulation of Cancer Genes by the RNA Binding Protein HuR. *AACR Annual Meeting (San Diego, April 12-16, 2008)*

Coordinate Posttranscriptional Regulation of Cancer Genes by the RNA Binding Protein HuR
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proteins (RBPs) is now beginning to be better appreciated. We study the *elav* (embryonic lethal abnormal vision) family of RBPs, which are paraneoplastic antigens, over-expressed in a variety of malignancies, including breast cancer. Antibodies against elav family members are believed to be cancer-protective. The *elav* family of RBPs binds to the AU-rich elements (AREs) found in the 3' untranslated regions (UTRs) of many early-response genes, including proto-oncogenes and cell cycle regulators. HuR, the ubiquitously expressed family member, has recently been described to play a role in cancer progression. HuR stabilizes and translationally up regulates the expression of its target mRNAs. Elevated levels of cytoplasmic HuR directly correlate with increased invasiveness of malignancy and poor prognosis for many cancers, including those of the breast. HuR has been described to control the expression of genes in all six different areas of transformation originally described by Hanahan and Weinberg. Hence, it has been recently suggested that HuR may serve as a tumor maintenance gene which allows for cancers, once they are established, to proliferate. Therefore, it is of interest to discover in vivo HuR targets, as these genes may play vital roles in transformed cells. We have developed methods which enable us to identify en masse, in vivo targets of RBPs such as HuR from cell lines and now for the first time, solid tissues as well. We call this ribonomic analysis, in which we immunoprecipitate subsets of ribonuclear particles (RNPs), exact the mRNA and then apply to microarrays. This technique, in which no cross linking is used, allows one to en masse identify different in vivo mRNA targets which different RBPs interact with. Using a combination of HuR immunoprecipitation and RNA interference, we have been able to identify HuR in vivo targets which may play important roles in tumor maintenance. Furthermore, we present evidence of cross talk amongst stabilizer and de-stabilizer RBPs which can potentially regulate the expression of multiple cancer targets. These techniques have the potential to better delineate genes whose steady state mRNA levels may not significantly change, but which are translationally active inside cancer cells. Potentially the identification of such genes may offer novel therapeutic targets to inhibit cancer growth.

(4) Magee, Joe, Gubin, Matt, Atasoy, Ulus. Coordinate Posttranscriptional Regulation of Cancer Genes by the RNA Binding Protein HuR. *Era of Hope CDRMP Meeting (Baltimore, June 25-28, 2008)*

Coordinate Posttranscriptional Regulation of Cancer Genes by the RNA Binding Protein HuR
Magee, Joe, Gubin, Matt, Atasoy, Ulus. University of Missouri, Columbia, Missouri.

Due to the poor correlation betw een steady stat e m RNA levels and protein products, trad itional microarray analysis m ay m iss m any genes which are regu lated primarily at the level of mRNA stability and translation. Posttranscriptional gene regulation is becoming increasi ngly recognized as an important form of cellular control. W hereas our understanding of transc riptional regulation is advanced, in contrast this area rem ains largely unexplored. Yet, increa singly, the im portance of microRNAs and RNA binding proteins (RBPs) is now beginning to be better appreciated. We study the *elav* (embryonic lethal abnormal vision) fam ily of RBPs, which are paraneoplastic antigens, over-express ed in a variety of m alignancies, including breast cancer. Antibodies against elav family members are believed to be cancer-protective. The *elav* family of RBPs binds to the AU-rich elements (AREs) found in the 3' unt ranslated regions (UTRs) of many early-response genes, including proto-oncogenes and cell cycle regulators. HuR, the ubiquitously expressed family member, has recently been described to play a role in cancer progression. HuR stabilizes and translationally up regulates the expression of its target mRNAs. El evated levels of cytoplasm ic HuR directly correlate with increased invasiveness of m alignancy and poor prognos is for m any cancers, including those of the breast. HuR has been descri bed to control the expres sion of genes in all six different areas of transform ation originally descri bed by Hanahan and W einberg. Hence, it has been recently suggested that HuR may serve as a tumor m aintenance gene which allows for cancers, once they are established, to proliferate. Therefore, it is of interest to discover in vivo Hu R targets, a s these genes may play vital roles in transform ed cells. W e have developed m ethods which enable us to identify en masse, in vivo targets of RBPs such as HuR fr om ce ll line s and now for the f irst tim e, solid tissues as well. W e call this ribonom ic analysis, in which we i mmunoprecipitate subset s of ribonuclear particles (RNPs), exact the m RNA and then apply to m icroarrays. T his technique, in which no cross linking is used, allows one to en ma sse identify different in vivo m RNA targets which different RBPs interact with. Using a combination of HuR i mmunoprecipitation and RNA interference, we have been able to identify HuR in viv o targ ets which m ay play im portant ro les in tu mor m aintenance. Fur thermore, we pres ent evidence of cross talk am ongst st abilizer and de-stabilizer RBPs wh ich can potentially regulate the expression of multiple cancer targets. These techniques have the potential to better delineate gene s whose steady state mRNA levels m ay not significantly change, but which are tr anslationally active inside cancer cells. Potentially the identification of such genes m ay offer novel therapeutic targets to inhibit cancer growth.

(5) Magee, Joe, Gubin, Matt, Atas oy, Ulus. RNA Immunopr ecipitations (RIP-on-Ch ip) Identify Unique Subsets of Genes in ER+ and ER- Breast Cancer. *AACR Annual Meeting (Denver, April 18-22, 2009; oral presentation)*

RNA Immunoprecipitations (RIP-on-Chip) Identify Unique Subsets of Genes in ER+ and ER- Breast Cancer

Magee, Joe, Gubin, Matt, Atasoy, Ulus, University of Missouri, Columbia, Missouri.

Due to the poor correlation betw een steady stat e m RNA levels and protein products, trad itional microarray analysis m ay m iss m any genes which are regu lated primarily at the level of mRNA stab ility and translation. Posttranscriptional gene regulation is becoming increasi ngly recognized as an important form of cellula r contro l. The im portance of m icroRNAs and RNA binding pr oteins (RBPs) is now beginning to be better appreciated. We study the *elav* (embryonic lethal abnormal vision) family of RBPs, which are p araneoplastic antig ens, over-expressed in a variety of m alignancies, including breast cancer. Antibodies against elav fa mily m embers are believed to be cancer-protective. The *elav* family of RBPs binds to the AU-rich elem ents (AREs) found in the 3' untranslated regions (UTRs) of m any early-response genes, including proto-oncogenes and cell cy cle regulators. HuR, the ubiquitously expressed family member, has been described to play a role in cancer progression. HuR stabilizes and translationally up regulates the expression of its target m RNAs. Elevat ed levels of cytoplasm ic HuR directly correlate with increased invasiveness of m alignancy and poor prognosis for m any cancers, including those of the breast. Recently, HuR has been implicated in tam oxifen resistance in breast cancer. HuR has been described to control the expression of genes in a ll s ix dif ferent areas of tran sformation origin ally described by Hanahan and W einberg. Hence, it has been suggested that HuR may serve as a tum or maintenance gene which allows for cancers, once they ar e established, to proliferat e. Therefore, it is of interest to discover in vivo HuR targets, as these genes may play vital roles in transformed cells. We have developed methods which enab le us to identify en m asse, in vivo ta rgets of RBPs such as HuR from cell lines and now for the first tim e, solid tissues as well. W e call this ribonomic analysis or **R**NA **i**mmuno**p**recipitations applied to m icroarrays, **RIP-on-Chip**. This technique, in which no cross linking is used, allows one to en ma sse identify different in vivo m RNA targets which different RBPs interact with. We have used RIP-on-Chip to identify distinct subsets of HuR associated m RNAs in MDA MB231 and MCF-7 breast cancer cell lines. W hereas some gene ta rgets are well app reciated cancer genes, a num ber do not have clear cut associations with cancer and may well represent novel targets. To further investigate the role of HuR in triple negative breast cancer, we have over expressed HuR in MB231 cells, which results in accelerated growth. Therefore, we conc lude that HuR pull downs can identify not only known but also novel cancer targets and RIP-on-Chip analysis identifies distinct subsets of cancer relevant genes.

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The RNA binding protein HuR differentially regulates unique subsets of mRNAs in estrogen receptor negative and estrogen receptor positive breast cancer

Robert Calaluce[1], Matthew M Gubin[1], J Wade Davis[2,3], Joseph D Magee[1], Jing Chen[1], Yuki Kuwano[6], Myriam Gorospe[6], Ulus Atasoy[1,4,5*]

Abstract

Background: The discordance between steady-state levels of mRNAs and protein has been attributed to posttranscriptional control mechanisms affecting mRNA stability and translation. Traditional methods of genome wide microarray analysis, profiling steady-state levels of mRNA, may miss important mRNA targets owing to significant posttranscriptional gene regulation by RNA binding proteins (RBPs).

Methods: The ribonomic approach, utilizing RNA immunoprecipitation hybridized to microarray (RIP-Chip), provides global identification of putative endogenous mRNA targets of different RBPs. HuR is an RBP that binds to the AU-rich elements (ARE) of labile mRNAs, such as proto-oncogenes, facilitating their translation into protein. HuR has been shown to play a role in cancer progression and elevated levels of cytoplasmic HuR directly correlate with increased invasiveness and poor prognosis for many cancers, including those of the breast. HuR has been described to control genes in several of the acquired capabilities of cancer and has been hypothesized to be a tumor-maintenance gene, allowing for cancers to proliferate once they are established.

Results: We used HuR RIP-Chip as a comprehensive and systematic method to survey breast cancer target genes in both MCF-7 (estrogen receptor positive, ER+) and MDA-MB-231 (estrogen receptor negative, ER-) breast cancer cell lines. We identified unique subsets of HuR-associated mRNAs found individually or in both cell types. Two novel HuR targets, *CD9* and *CALM2* mRNAs, were identified and validated by quantitative RT-PCR and biotin pull-down analysis.

Conclusion: This is the first report of a side-by-side genome-wide comparison of HuR-associated targets in wild type ER+ and ER- breast cancer. We found distinct, differentially expressed subsets of cancer related genes in ER+ and ER- breast cancer cell lines, and noted that the differential regulation of two cancer-related genes by HuR was contingent upon the cellular environment.

Background

Over the past decade array technologies have provided several new means for profiling global changes in gene expression. The power of DNA microarrays is perhaps best illustrated in the way it has been used to differentiate treatment responses in patient populations. Individualized and targeted therapy for several tumors, based upon underlying differences at the molecular level among gene expression profiles, is beginning to replace the traditional morphological-based treatment paradigm [1-3]. Genome wide microarray analyses, however, are inherently flawed since they globally profile the steady-state levels of mRNA, referred to as the transcriptome. Cellular protein expression levels, however, do not directly correlate with steady-state levels of mRNAs. It is well accepted in the RNA field that there is a poor correlation between steady-state RNA levels and protein.

* Correspondence: atasoyu@missouri.edu
[1]Departments of Surgery, University of Missouri, One Hospital Drive, Columbia, Missouri 65212, USA

This discordance has been attributed to posttranscriptional control mechanisms affecting mRNA stability and translation. Steady-state mRNA levels of genes, controlled partially or totally at this level, may be misleading. Gygi and colleagues have shown that correlation between mRNA and protein levels could not be predicted from only mRNA steady-state levels [4]. They observed that some genes had the same mRNA levels but protein levels varied more than 20 fold. Conversely, some proteins were of equal expression but their respective mRNA level varied by more than 30-fold. They concluded that "transcript levels provide little predictive value with respect to the extent of protein expression" [4]. Additionally, Idekar and colleagues have described similar results for the galactose gene [5].

Although our understanding of transcriptional gene regulation is advanced, posttranscriptional gene regulation remains largely unexplored. It is becoming clear, however, that this is an important mode of gene regulation, especially for proinflammatory genes. These genes appear to be posttranscriptionally regulated by RNA binding proteins (RBPs) which interact with AU-rich elements (AREs) in the 3' untranslated region (UTR) of mRNAs. Approximately 3,000 human genes contain AREs, representing 8% of the human genome [6]. Many of these genes which possess AREs are in areas of transient biological responses, including cell growth and differentiation, immune responses, signal transduction, transcriptional and translational control, hematopoiesis, apoptosis, nutrient transport, and metabolism [6,7].

New methodologies have provided global identification of *in vivo* mRNA targets of different RBPs. One of these, termed the ribonomic approach, involves the immunoprecipitation of ribonucleoprotein complexes (RNPs) with antibodies against different RBPs, extraction of mRNA, and hybridization to microarrays [8-10]. This approach, also referred to as RIP-Chip, enables investigators to identify groups of posttranscriptionally regulated mRNAs coordinately controlled by RBPs during various biological processes. A new paradigm, the posttranscriptional operon hypothesis, has been developed which states that RBPs coordinately regulate the expression of biologically related molecules [11,12]. This paradigm is being confirmed by the work of many different laboratories as our understanding of posttranscriptional regulation broadens and putative operons are described [8,13-17]. HuR is an RBP that binds to AREs of many proto-oncogenes and labile mRNAs. It has emerged as a key regulatory factor which stabilizes and translationally enhances its targets mRNAs, and affects their transport from the nucleus to the cytoplasm [18-20]. HuR belongs to the *ELAV* (*embryonic lethal abnormal vision*) family found in mammalian cells containing four members: HuR, HuB, HuC, and HuD. HuR is the only ubiquitously expressed member. The other family members are found primarily in the central nervous system and gonadal tissue [18]. Many HuR targets are cytokines, chemokines, and other early-response genes [21,22].

Of the hallmarks of cancer originally described by Hanahan and Weinberg, HuR has been demonstrated to control expression of genes in multiple areas of malignant transformation [23]. Consequently, HuR has been suggested to function as a tumor maintenance gene, permissive for malignant transformation, tumor growth, and perhaps metastasis [24]. HuR has been described in the literature as controlling the expression of many cancer-relevant genes, including those that encode these proteins: Prothymosin-α, Bcl-2, Mcl-1, SirT1, TGF-β, MMP-9, MTC-1, uPA, VEGF-α, HIF1-α and cyclins A1 (CCN A1), B1 and D1 [25-35]. Increased levels of HuR have been associated with a more aggressive breast cancer and a worse prognosis [36-38]. Of significance, HuR has been described as posttranscriptionally regulating the expression of many breast cancer relevant genes including those that encode Glut-1, ERα, COX-2, IL-8, Cyclin E1, and most recently BRCA-1 [36,39-44]. HuR RIP-Chip analysis has recently identified Thrombospondin 1 as a key HuR target in the MCT-1 transformed estrogen receptor positive (ER+) cell line MCF-7 [45]. Its interactions, however, are complex and, at times, HuR may interact with miRNAs such as Let-7 to translationally suppress the expression of *C-MYC* mRNA [46].

Since HuR has been described as regulating the expression of many cancer relevant genes, we asked whether it may coordinately regulate breast cancer genes in ER+ and ER- breast cancer. We performed a HuR RIP-Chip analysis on MDA-MB-231 (ER-) and MCF-7 (ER+) cell lines to identify cancer-relevant genes, not known to be regulated by HuR, and potential novel breast cancer targets. Our studies indicated that HuR was associated with unique subsets of mRNAs in each cell line as well as a subset of HuR associated mRNA targets common to both. We chose two cancer-associated genes, *CD9* and *CALMODULIN 2* (*CALM2*), highly expressed in both cell lines, and functionally validated the role of HuR in regulating their expression. Unexpectedly, HuR differentially regulated the same target, *CD9*, in both cell lines in an opposite manner. Moreover, we found presumptive differential regulation of *CALM2* by HuR, as HuR interacted only with *CALM2* mRNA, but not with family members *CALM1* and *CALM3* mRNAs. We discovered that HuR interacts with many breast cancer-relevant genes not previously known to be controlled by HuR, and target genes which have not been shown to be cancer related. This latter category may indeed represent novel cancer genes discovered by HuR RIP-Chip analysis.

Methods

Cells in culture

The MDA-MB-231 (MB-231) and MCF-7 cell lines were obtained from American Type Culture Collection (Manassas, VA). The cell lines were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. MB-231 cells were grown in RPMI (GIBCO®, Invitrogen™, Carlsbad, CA) containing 10% fetal calf serum (Hyclone, Thermo Fisher Scientific, Waltham, MA), 0.5 mM L-glutamine (GIBCO®), 25 mg/ml glucose (Sigma-Aldrich), HEPES (GIBCO®) and sodium pyruvate (GIBCO®). MCF-7 cells were grown in DMEM (GIBCO®) supplemented with 10% fetal calf serum.

HuR Immunoprecipitations (RIP-Chip)

HuR RIP-Chip was performed as previously described [8,47,48]. Briefly, lysates were prepared from exponentially growing MB-231 and MCF-7 cells. Equal amounts of protein lysates were used (100-300 μg). HuR monoclonal antibody 3A2 (made in our laboratory from the 3A2 hybridoma, generously provided by Dr. Joan Steitz, Yale University, New Haven, CT), or isotype control IgG1 (BD Biosciences, San Jose, CA), were pre-coated onto Protein A Sepharose beads (PAS) and extensively washed. Lysates from each cell initially were pre-absorbed with 30 μg of IgG1 and then removed by addition of PAS beads. Individual pull-downs were performed at 4°C for only 1-2 hr to minimize potential re-assortment of mRNAs.

RNA amplification

The entire amount of recovered RNA per immunoprecipitation was amplified using the WT-Ovation™ Pico RNA Amplification System protocol (NuGen, San Carlos, CA). Forty ng of total RNA was used as starting material to generate at least 6 μg of cDNA. Amplified cDNA was purified using Zymo Research Clean and Concentrator™-25 (Zymo Research, Orange, CA). Three μg of amplified and purified cDNA was incubated at 50° C for 30 minutes with 5 μl of UNG buffer and 5 μl UNG enzyme and 60 minutes with 5 μl labeling buffer and 5 μl ARP (biotin) solution as described in NuGen's labeling protocol for the Illumina BeadArray platform. All samples (total RNA, amplified cDNA, and biotin labeled amplified cDNA) were quantitated using a Nanodrop™ (Thermo Fisher Scientific, Waltham, MA) spectrophotometer. RNA quality and integrity were assessed on selected samples with the Experion™ automated electrophoresis system (Bio-Rad, Hercules, CA).

Microarray

Biotin-labeled, amplified cDNA (1.5 μg) was hybridized to a Sentrix® Human-6 v.2 Whole Genome Expression BeadChips (Sentrix Human WG-6; Illumina, San Diego, CA). Each chip tested 6 samples and contained 47,293 gene targets, representing 18,025 distinct RefSeq genes that are not pseudogenes. A total of 3 chips were used for this experiment. The chips were hybridized at 48°C for 20 hr in the hybridization buffer provided by the manufacturer. After hybridization, the chips were washed and stained with streptavidin-C3. The chips were scanned on the BeadArray Reader, as described by Illumina at http://www.illumina.com. The Illumina BeadStudio software was used to assess fluorescent hybridization signals.

Quantitative RT-PCR

Selected genes were validated by quantitative RT-PCR. Briefly, cDNA was generated from the same samples as previously described for the microarray experiments using 10 ng total RNA and the SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen Carlsbad, CA). RT-PCR was performed on the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Each sample was run in triplicate for these genes and the cDNA was divided equally per reaction in a 20 μl volume. The PCR conditions were: 50°C for 2 minutes and 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds alternating with 60°C for 30 seconds. Melting curve analysis was performed on every reaction to confirm a single amplicon. For each cell line, differences in gene expression were determined using the equation $2^{-\Delta\Delta Ct}$, where the C_t value for either the HuR or IgG IP was subtracted from the C_t value of the *GAPDH* control to yield the ΔC_t value. For each cell line, the ΔC_t value for the HuR and IgG IP were computed in triplicate and averaged to give one $\Delta\Delta C_t$ value per sample. Primers used:

Human RT *GAPDH* Forward 5' AGCCTCAAGATCATCAGCAATGCC 3'

Reverse 5' TGTGGTCATGAGTCCTTCCACGAT 3'

Human RT *HuR* Forward 5' ATGAAGACCACATGGCCGAAGACT 3'

Reverse 5' AGTTCACAAAGCCATAGCCCAAGC 3'

Human RT *CD9* Forward 5' TCAGACCAAGAGCATCTTCGAGCA 3'

Reverse 5' ACCAAGAGGAAGCCGAAGAACAGT 3'

Human RT *CALM2* Forward 5' CTGACCAACTGACTGAAGAGCAGA 3'

Reverse 5' TTCTGTGGGATTCTGCCCAAGAG 3'

Cloning strategy of HA HuR

Hemagglutinin (HA) tagged human HuR was cloned into the *NheI* and *XhoI* sites of the pZeoSV2 (-) vector (Invitrogen). The plasmids were sequenced in both directions to confirm identity. Cells were transfected

with either pZeo HA HuR or pZeo empty vector using Lipofectamine 2000 (Invitrogen). After five days transfected media was removed and replaced with fresh medium containing 200 μg/ml of Zeocin antibiotic (Invitrogen). Cells were selected for a ten day period. After ten days, the selected cells were maintained in 50 μg/ml of Zeocin to maintain pZeo HA HuR and empty vector expression. No viable cells remained in the untransfected well. Cells were then cloned by limiting dilution.

Lentiviral RNAi HuR knock-down

In order to knockdown HuR, PSICOOLIGOMAKER v1.5 http://web.mit.edu/ccr/labs/jacks/was used to identify optimal shRNAs sequences to HuR. We tested multiple sequences and chose GGATCCTCTGGCAGATGT, identified and designated shRNA H760. Annealed sense and antisense DNA (Integrated DNA Technologies, Inc, IDT, Coralville, IA), along with stem loops to create hairpin, were cloned into the *HpaI* and *XhoI* restriction sites in the Lentilox pll3.7 vector (ATCC). After sequence verification, lentivirus was packaged in 293FT cells using ViraPower™ Lentiviral Expression Systems (Invitrogen) following manufacturer's protocol. Both MB-231 and MCF-7 cells were seeded at a density of 100,000 cells in 100 mm tissue culture plates with 10 ml of media. The following day lentivirus, expressing either GFP and no shRNA (empty lentilox control) or GFP and HuR shRNA H760, was added at a multiplicity of infection (MOI) of 10 along with polybrene (8 μg/ml) (Sigma-Aldrich Corp, St. Louis, MO). After five days, cells were harvested by trypsinization and sorted for GFP expression using BD FACSDiva (BD Bioscience). Cells were cloned by limiting dilution and GFP expression was assessed using FACScan (BD Bioscience) and CellQuest software (BD Bioscience). GFP expression was >98% and indicated homogenous cell population.

SDS-PAGE and Western Blot Analysis

Western analysis was performed as described previously with slight modifications [47]. Briefly, cells were harvested and lysed in triple-detergent RIPA buffer with protease inhibitor cocktail (Roche, Pleasanton, CA). For nuclear and cytoplasmic fractionation, the NE-PER kit was used (Pierce, Rockford, IL). Protein quantity was determined by Bradford Assay. Forty μg of protein was electrophoresed on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk at room temperature for 1 hr and incubated with anti-β-tubulin (1 μg/ml, Sigma-Aldrich) at 4°C overnight. After washing, the membrane was incubated with monoclonal anti-HuR clone 3A2 antibody (1 μg/ml) at room temperature for 1 hr, or anti-CD9 antibody (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C overnight. The secondary antibody used was sheep anti-mouse Ig horse radish peroxidase (1:4000) (GE Healthcare, Piscataway, NJ), incubated at room temperature for 1 hr. Specific proteins were detected using chemiluminescence (GE Healthcare). HuR knock-down was determined to be >90% using Bio-Rad's Quantity One software (Bio-Rad) normalizing to β-tubulin, and HuR over-expression was quantitated in a similar manner.

Biotin Pull-down

Biotinylated transcripts were synthesized using cDNA that was prepared from MB-231 cells. Templates were prepared using forward primers that contained the T7 RNA polymerase promoter sequence (CCAAGCTTCTAATACGACTCACTATAGGGAGA [T7]). Primers used for the preparation of biotinylated transcripts spanning the *CD9* CR, and 3'UTR (NM_001769) and *CALM2* CR and 3'UTR (NM_001743.3) were as follows:

CD9 CR 118-560: [T7] TCAAAGGAGGCACCAAGTGCAT and AACGCATAGTGGATGGCTTTCA

*CD9*3'UTR798-1231: [T7] AGTCAGCTTACATCCCTGAGCA and GACATTGTCATAATTTTTTATTATGTATC

CALM2 CR 72-515: [T7] GCTGACCAACTGACTGAAGA and CTTTGCTGTCATCATTTGTACAAA

CALM2 3'UTR 518-1128: [T7] AGACCTTGTACAGAATGTGTTAA and GGGTAAATTGTAATTTTTTTATTGGAA

GAPDH 3'UTR: [T7] CCTCAACGACCACTTTGTCA and GGTTGAGCACAGGG TACTTTATT

The PCR-amplified fragments were purified and used as templates for *in vitro* synthesis of the corresponding biotinylated RNAs by MAXIscript kit (Ambion®, Applied Biosystems). Biotin pull-down assays were performed by incubating 40 μg of MB-231 cell lysates with equimolar of biotinylated transcripts for 1 hr at room temperature. The complexes were isolated using paramagnetic streptavidin-conjugated Dynabeads (Dynal®, Invitrogen), and bound proteins in the pull-down material were analyzed by Western blotting using an antibody recognizing HuR (Santa Cruz). After secondary-antibody incubations, the signals were visualized by chemiluminescence (Amersham Biosciences, GE Healthcare).

Statistical Analysis of Microarray Data

Analysis of microarray gene expression data was primarily performed using the Linear Models for Microarray Data (limma) package [49] and the lumi package [50], available through the Bioconductor project [51] for use with R statistical software [52]. After data pre-processing was completed (Appendix), the statistical analysis was performed using moderated *t*-statistics applied to the

log-transformed (base 2) normalized intensity for each gene using an Empirical Bayes approach [53]. Three contrasts of interest were computed and tested. The first was the difference between HuR pull-down and IgG background for the MB-231 cell line. Genes which exhibited significantly greater expression in the pull-down were considered to be in the HuR pellet for the MB-231 cell line. The second contrast was similar to the first, but for the MCF-7 cell line. The third and most important contrast was the difference between the first and second contrast, and can be viewed as a test of statistical interaction between HuR and cell line. For a given gene, this term can be interpreted as reflection of the synergistic relationship between HuR and estrogen in breast cancer. Adjustment for multiple testing was made using the false discovery rate (FDR) method of Benjamini and Hochberg [54] with an FDR of 10% as our cutoff for declaring significance. To facilitate interpretation, log fold changes were transformed back to fold change on the data scale (fluorescent intensity).

Gene ontology (GO) analyses were carried out on the list of significant genes based on the third contrast described above. The purpose of the analyses was to test the association between Gene Ontology Consortium categories [55] and differentially expressed HuR pellet genes between MB-231 and MCF-7. Using our defined gene universe (Appendix), GOstats [56] was used to carry out conditional hypergeometric tests. These tests exploit the hierarchical nature of the relationships among the GO terms for conditioning [57]. We carried out GO analyses for over-representation of biological process (BP), molecular function (MF), and cellular component (CC) ontologies, and computed the nominal hypergeometric probability for each GO category. These results were used to assess whether the number of selected genes associated with a given term was larger than expected under the null hypothesis, and a p-value cutoff of 0.01 was used. GO categories containing less than 10 genes from our gene universe were not considered to be reliable indicators, and are not reported.

Results

HuR immunoprecipitation from ER+ and ER- breast cancer cell lines

We first determined HuR protein expression levels in breast cancer cell lines. HuR is expressed in both the ER- and the ER+ cell lines, MB-231 and MCF-7, respectively (Figure 1A). RNA immunoprecipitation, using HuR monoclonal antibody 3A2, recovered HuR (Figure 1A) and revealed, by quantitative RT-PCR, a significant enrichment of up to fifteen fold for a known HuR target, *β-ACTIN* mRNA, as compared to isotype control (IgG1) and normalized to a non-target, *GAPDH* mRNA (Figure 1B). These data showed that HuR RIP specifically immunoprecipitate HuR protein and associated mRNAs, though absolute quantitative conclusions cannot be drawn since different amounts of lysates were used and efficiency of immunoprecipitation from different cell lines may differ.

RIP-Chip from ER+ and ER- breast cancer cell lines identifies unique sets of associated mRNAs

RIP-Chip was performed on cytoplasmic lysates from both breast cancer cell lines with HuR antibody and isotype control in order to determine HuR associated mRNAs. Each immunoprecipitation was done at least three independent times with matching controls. Signals from isotype control were subtracted out. Recovered mRNA was amplified and hybridized to Illumina Sentrix Human arrays consisting of 47,000 genes. Figure 2 represents a composite array generated by combining hybridizations to twelve different arrays ($\log_2$ scale). Three groups of HuR-associated target genes were identified: MB-231 targets in the left upper quadrant; both MB-231 and MCF-7 targets in the right upper quadrant; MCF-7 targets in the right lower quadrant. As expected, most of the mRNAs did not associate with HuR and were located in the lower left quadrant. There were 395 and 64 annotated genes, at least 2 fold or more enriched, associated with either MB-231 or MCF-7 cells, respectively, and 182 genes associated with both cell lines. A complete list can be found in Additional File 1, Figure S2. The raw data files are available in the NCBI database at the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pdsnrqmiawukql-m&acc=GSE17820, NCBI Accession number GSE17820). These genes generally fell into three groups. Group 1 consisted of cancer-associated genes which were known HuR targets, such as *PTMA* mRNA. Group 2 consisted of genes which played a role in cancer but were not known to be HuR targets. Group 3 consisted of genes with an unknown function in cancer, but which may be regulated by HuR. These data revealed that HuR was associated with distinct subsets of mRNAs in ER+ and ER- breast cancer cells.

Gene Ontology (GO) analyses of differentially expressed significant genes between ER+ and ER- cells were categorized into Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). GO analyses allow for the identification of gene families that may play significant roles related to these categories in expression profiles. Most of the differentially expressed genes (155) were found to be more abundant than expected in 14 BP categories (Figure 3A). Three MF categories consisted of 100 genes with most of these (83) related to protein binding and transcription activator activity. The CC categories contained the least (34) and were primarily associated with the Golgi apparatus.

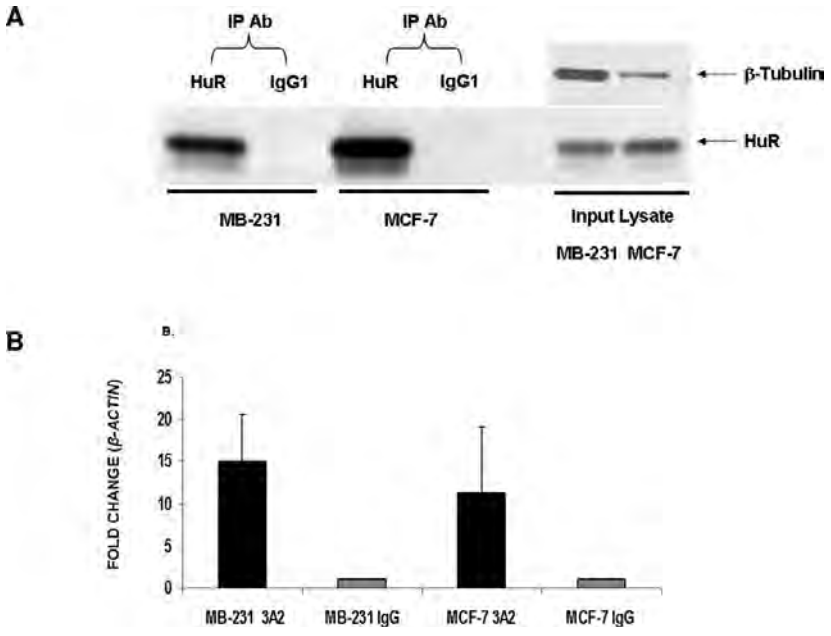

Figure 1 Immunoprecipitation and RIP in MB-231 and MCF-7 breast cancer cells. Immunoprecipitations were performed from MB-231 or MCF-7 cell lysates using anti-HuR monoclonal antibody (3A2) and IgG1 isotype control. **A**. IP Western of HuR revealed expected size band as detected by 3A2. Panel on right reveals amounts of HuR in lysates used from both cell lines. **B**. Verification by quantitative RT-PCR showed fifteen and eleven fold enrichments of *B-ACTIN*, a known HuR target, in the 3A2 IPs from MB231 and MCF-7, respectively. All $\Delta\Delta C_T$ values were normalized to *GAPDH*. Experiments were done in duplicate (n = 2).

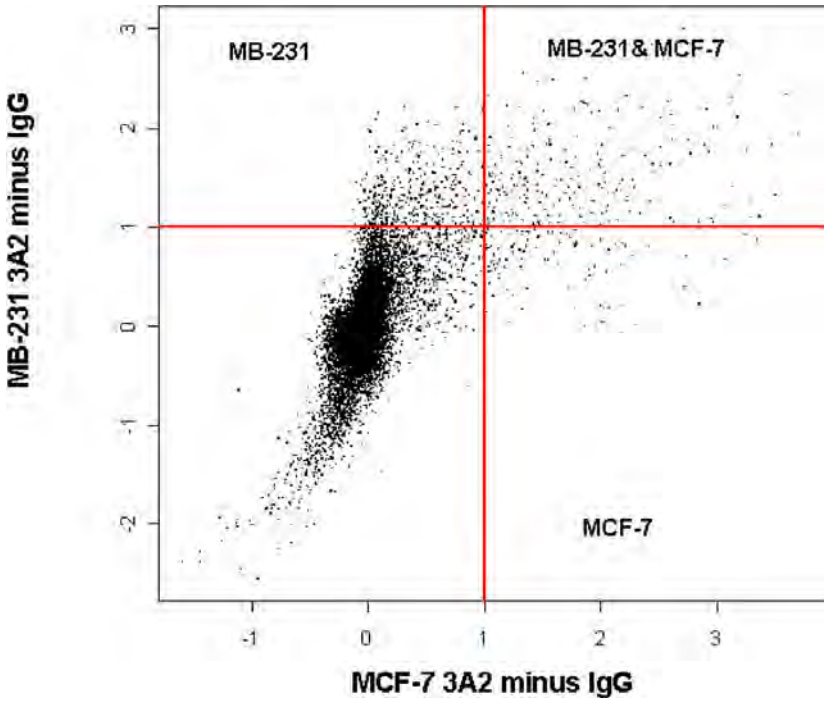

Figure 2 HuR RIP-CHIP identifies distinct genetic profiles in ER+ and ER- breast cancer cells. HuR immunoprecipitations were performed from MB-231 or MCF-7 cell lysates using HuR antibody and IgG1 isotype control hybridized to Illumina Sentrix arrays (47,000 genes). Control signals were subtracted. Results represent cumulative data from 12 different arrays. Experiments were done in triplicate (n = 3) for each cell line with matching controls. Scales are $\log_2$.

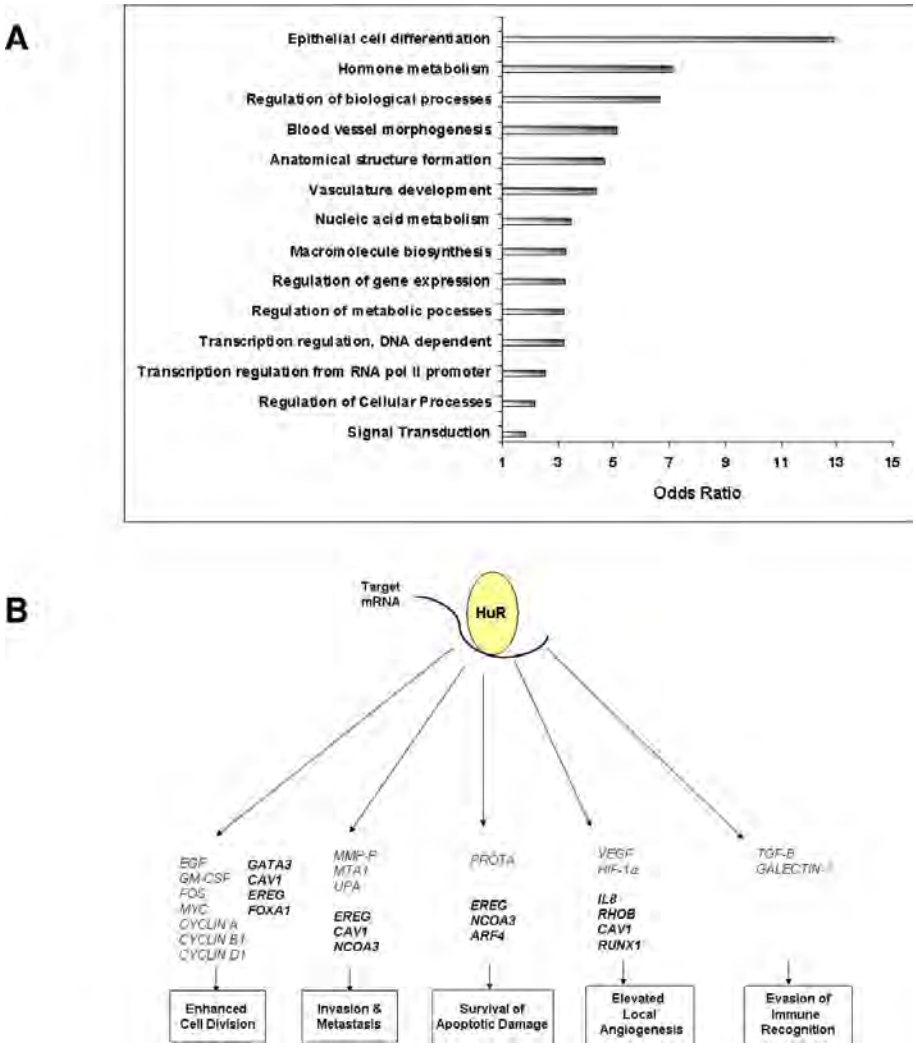

Figure 3 GO Classification of genes found by RIP CHIP of potential HuR targets and their relationship to the Acquired Capabilities of Cancer Model. **A**. Differentially expressed genes which are more represented in the Biological Processes (BP) GO category than expected. **B**. Original representation showing subsets of transcripts found to be targets of association with HuR (normal type). New transcripts found in this study with RIP-Chip (bold type). Enhanced expression upon binding to HuR influences several of the acquired capabilities of cancer cells described by Hanahan and Weinberg [23,24].

For the complete GO analyses see Additional File 2, Table S1. In Table S1 we list the top HuR associated mRNAs in the different categories which were approximately 5 fold enriched or greater. As can be seen in Figure 3B, a partial listing of some of these genes (in bold) are candidate members to multiple areas of cancer control, as suggested by Hanahan and Weinberg. We note that though β-*ACTIN* mRNA was amongst the most abundant of HuR-associated mRNAs in MCF-7 cells, β-*ACTIN* mRNA levels were only 3.93-fold higher in HuR IP compared to IgG IP in MB-231 cells. Therefore, since this was less than the 5-fold cut-off we employed for Table S1, it is not listed. Thus, these results may have identified novel HuR-controlled genes which may play roles in breast carcinogenesis in a cancer subtype-specific fashion.

Validation of HuR targets CD9 and CALM2 by real-time PCR and biotin pull-down analyses

In order to validate HuR binding to genes identified in Figure 2, we chose two known cancer associated genes, *CD9* and *CALM2*, highly expressed in both cell lines. Two independent approaches confirmed the physical interaction between HuR, *CD9* and *CALM2* mRNAs. Precipitated mRNA from the RIP-Chip experiments were analyzed by quantitative RT-PCR. Both *CD9* and *CALM2* mRNAs were enriched in the HuR RIP by as much as 160 fold (Figures 4A and 4B), but not the isotype control IP. We

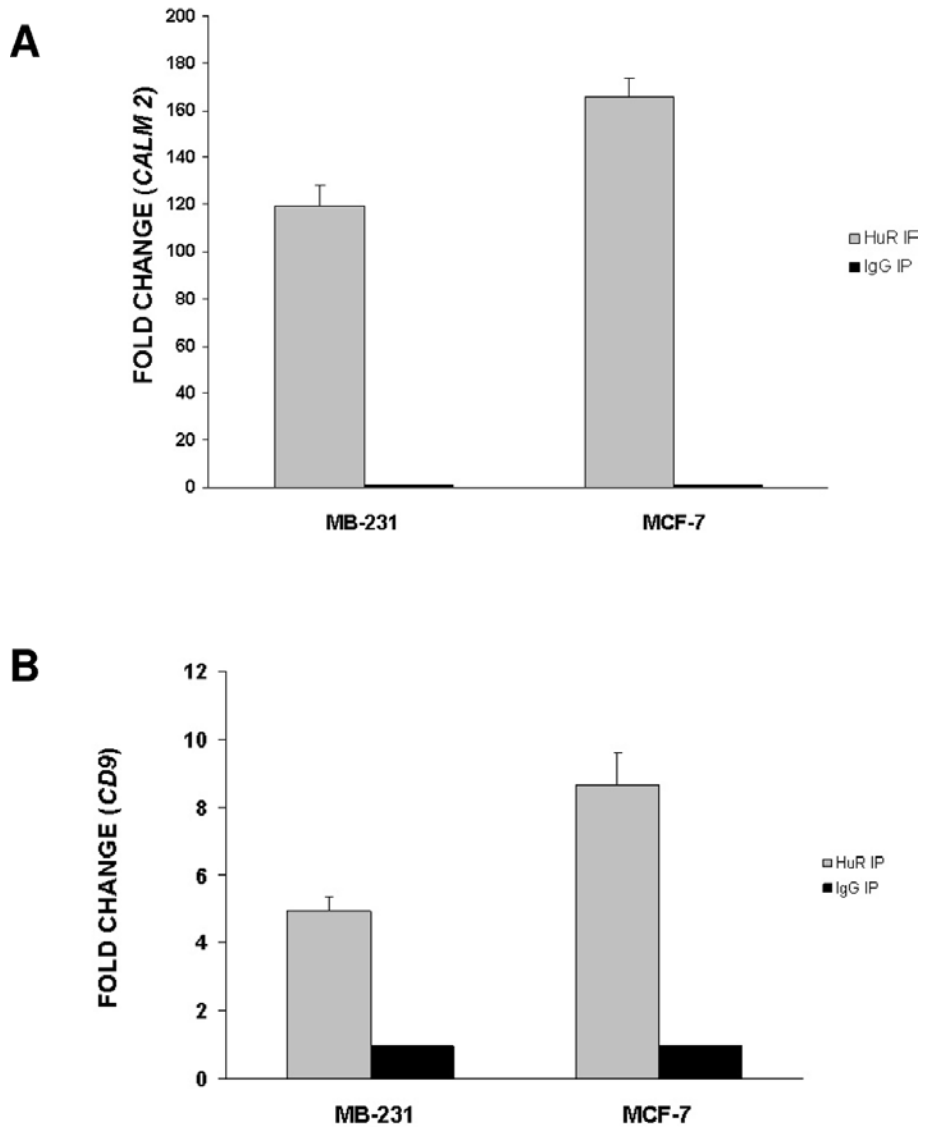

Figure 4 Validation of target *CALM2* and *CD9* mRNAs by quantitative RT-PCR. Quantitative RT-PCR using cell lysates, HuR antibody, and IgG1 from RIP-CHIP analysis confirmed results identifying *CALM2* mRNA **(A)** and *CD9* mRNA **(B)** as HuR targets. Change in gene expression is represented as fold increase in HuR immunoprecipitation as compared to IgG1. *GAPDH* mRNA was used as an endogenous control. Error bars represent SEM. p value is < 0.005. Experiments were done in triplicate (n = 3).

further confirmed HuR binding to *CD9* and *CALM2* mRNAs by biotin pull-downs. The relevant portion of the mRNA was transcribed with biotin tags and incubated with lysates from the two cell lines to probe for interactions with protein. The mixture was then separated by pull-down using streptavidin-coated beads and HuR levels were analyzed by Western blot analysis. As seen in Figure 5, HuR specifically interacts with *CD9* and *CALM2* mRNAs in the 3'UTR regions, but not within the coding region (CR) or with a control biotinylated RNA corresponding to the 3'UTR of the housekeeping control *GAPDH* mRNA, which is not a target of HuR.

HuR differentially regulates CD9 and CALM2 in MB-231 and MCF-7 cell lines

To gain insight into the biological effects of these associations, we studied the consequences of stably increasing or decreasing HuR abundance. Individual MB-231 clones which over- and under-express HuR were established by limiting dilution (Figure 6A and 6B). MB-231 cells over-expressed HuR by about 140% (Figure 6A). HuR knock-down using lentiviral shRNA resulted in ~95% reduction in HuR expression (Figure 6B). Surprisingly, over-expression of HuR in MB-231 cells caused decreases in both *CD9* protein and mRNA levels

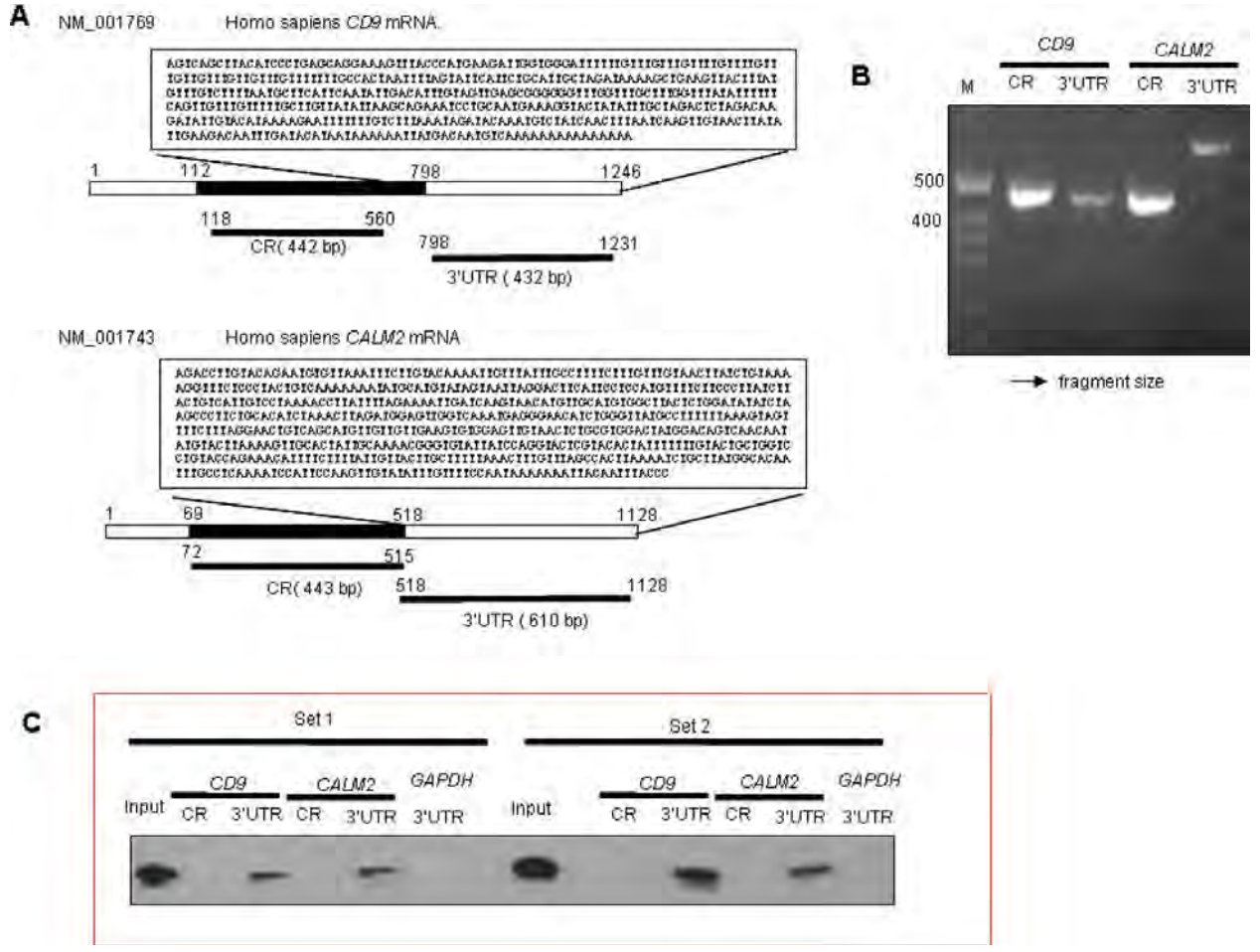

Figure 5 Biotin Pull-down of *CD9* and *CALM2*. A. Scheme of Coding region (CR) and 3'UTR fragments for biotin pull-down assay. The sequences were obtained from Entrez data base. CR and 3'UTR fragments selected for amplification by PCR are as noted. **B**. 1% agarose gel electrophoresis showing PCR amplified products of the coding regions and 3'UTR's for *CD9* (442 bp and 432 bp, respectively) and *CALM2* (443 bp and 610 bp, respectively). **C**. Biotin pull-down assay using lysates prepared from MB-231 cells. The binding of HuR to biotinylated 3'UTR transcripts from both *CD9* and *CALM2* mRNAs was specific. HuR did not bind a biotinylated control (*GAPDH* 3'UTR); and did not bind to biotinylated transcripts spanning the CR of *CD9* or *CALM2*. Experiments were done in duplicate (n = 2).

(Figures 6C and 6D). HuR knock-down, however, resulted in increases in both *CD9* mRNA and protein levels (Figures 6C and 6E). This is the opposite of what we predicted, since HuR is generally regarded as a stabilizer of mRNA. In contrast, over-expression of HuR in MB-231 cells did not significantly alter the levels of *CALM2* mRNA (Figure 6D). Figure 6F depicts a graphical analysis which reveals that HuR over-expression decreases both *CD9* mRNA and protein levels, as compared to controls (dashed line set at 100%). Whereas, HuR shRNA knock-down results in increases in both *CD9* mRNA and protein levels above control levels.

We performed similar analyses with MCF-7 cells, though the over-expression levels of HA HuR were only approximately 10%, since this was a pooled population (we have been unable to obtain MCF-7 clones which over-express HuR). In contrast, we generated MCF-7 clones with reduced HuR levels (93%) using lentiviral shRNA (Figure 7B). Western blot analysis of MCF-7 cells with over-expression of HuR reveals modest increases in *CD9* protein levels (Figure 7C). There are also modest decreases in *CD9* protein expression in MCF-7 with reduced HuR levels (Figure 7C). mRNA levels of *CD9* and *CALM2* are essentially unchanged in MCF-7 cells which over-express HuR (Figure 7D). As expected, HuR knock-down in MCF-7 cells using lentiviral shRNA resulted in significant reductions in both *CD9* and *CALM2* mRNA levels (Figure 7E). The right panel in Figure 7E indicates efficiency of HuR mRNA knock-down which is consistent with the protein data (Figure 7B). These results are summarized in Figure 7F. There are no significant changes seen in *CD9* mRNA

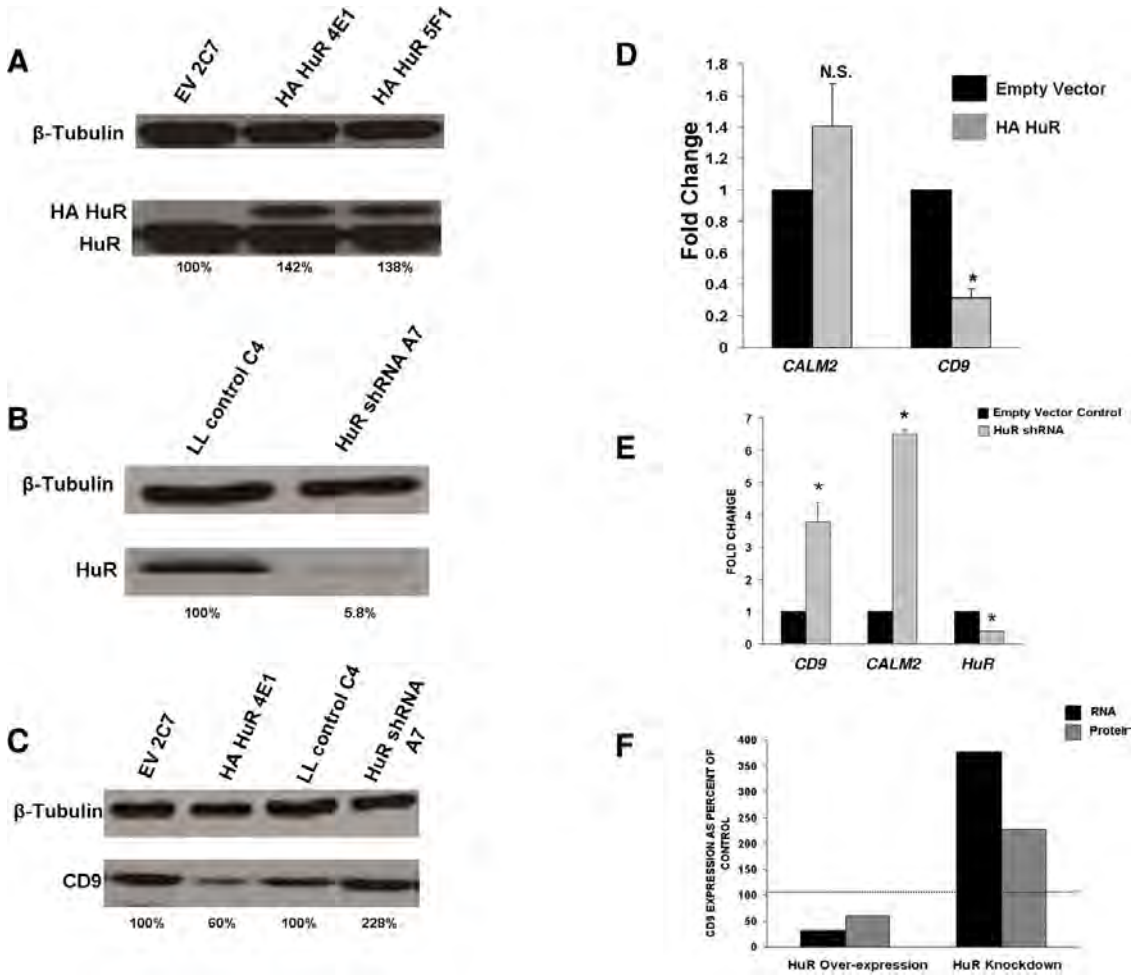

Figure 6 HuR differentially regulates *CD9* and *CALM2* in MB-231. A. Epitope HA tagged HuR is over-expressed by 142% and 138% respectively, in stably transfected clones 4E1 and 5F1, as compared to empty vector (EV) control clone 2C7. **B**. HuR knock-down using lentiviral short hairpin (sh) RNA H760 results in a 94% reduction in steady state levels of protein in clone A7 (LL = lentilox control). **C**. HuR over-expression results in a 40% reduction in CD9 protein levels as assayed by Western analysis; however, HuR knock-down using lentiviral shRNA results in an increase from 100% to 228% of *CD9* levels. **D**. Over-expression of HuR decreases *CD9* mRNA levels but not *CALM2* expression. Analysis of steady state *CD9* and *CALM2* mRNA levels by quantitative RT-PCR reveals significant decreases in *CD9* mRNA levels, whereas *CALM2* levels are unaffected. Although *CALM2* expression appears greater, the change is not significant. **E**. Knocking down HuR levels by shRNA in MB-231 cells shows significant increases in *CD9* and *CALM2* mRNA levels by quantitative RT-PCR. Decreased levels of HuR mRNA validate HuR shRNA knock-down. **F**. Graph showing the effects of HuR on the expression of *CD9* mRNA. HuR over-expression results in decreases in both mRNA and protein levels, though the decreases are greater in RNA. Whereas, HuR knock-down by shRNA results in significant increases at both the mRNA and protein levels, with greater change at transcript levels. The dashed line represents levels in control cells. Error bars represent SEM. p value is < 0.005; N.S. = not statistically significant; * = statistically significant. All experiments were done in triplicate (n = 3).

and CD9 protein for HuR over-expression. There is a more pronounced knock-down, however, in *CD9* mRNA in MCF-7 cells with reduced HuR levels.

The results of HuR shRNA knock-down in MCF-7 cells were as expected, but opposite of those seen for MB-231 cells. Steady-state mRNA levels of *CD9* and *CALM2* mRNAs decreased, consistent with the hypothesis that HuR generally stabilizes its mRNA targets. One possible explanation of these disparate results is different levels of total cellular or cytoplasmic HuR. We performed nuclear and cytoplasmic fractionation (Additional File 3, Figure S3). These results demonstrated modest (approximately 10%) greater cytoplasmic levels of HuR in MB-231 cells as compared to MCF-7. The total cellular HuR levels are very similar for both MB-231 and MCF-7 cells. Taken together, these results indicated that HuR appeared to differentially regulate the same mRNAs in a manner dependent upon the cellular milieu.

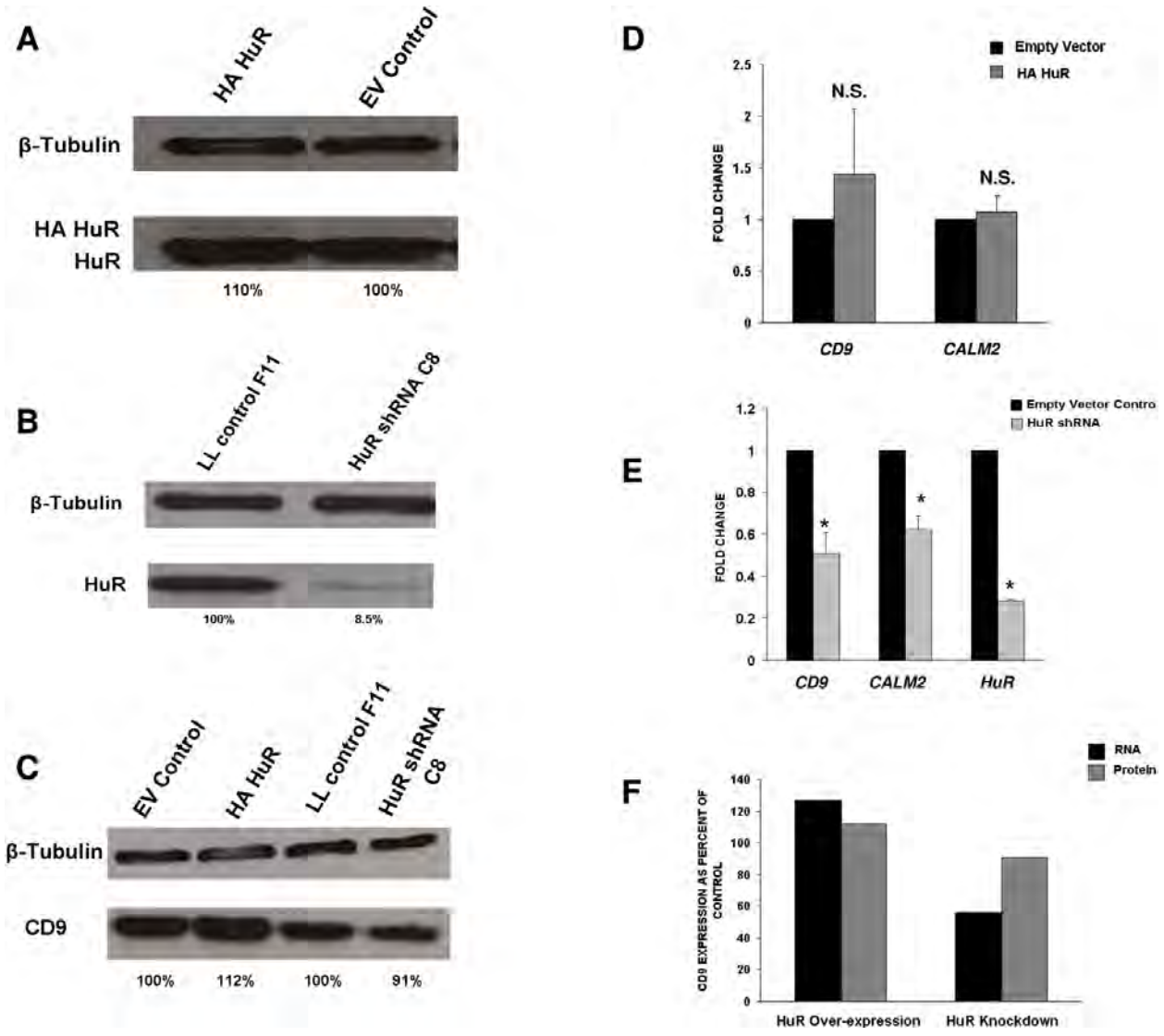

Figure 7 Effects of over-expressing or reducing HuR on *CD9* and *CALM2* expression in MCF-7 cells. A. Western analysis of HuR over-expression in heterogenous population of cells reveals approximately 10% over-expression. **B**. Lentiviral HuR shRNA efficiently knocks down HuR protein by over 90%. **C**. HuR over- expression and under-expression results in small changes in CD9 protein levels in MCF-7 cells. **D**. Levels of both *CD9* and *CALM2* mRNAs are unchanged in cells which over-express HuR; whereas lentiviral knock-down of HuR in MCF-7 cells results in decreases in steady-state mRNA levels **(E)**. The graph in **(F)** shows minimal changes in *CD9* mRNA and protein levels in HuR over-expressing MCF-7 cells. The *CD9* mRNA levels, however, are more affected in HuR knock-down. P value is < 0.005; N.S. = not statistically significant; * = statistically significant.

Discussion

We utilized RIP-Chip technologies to define differentially regulated HuR genes in ER+ and ER- breast cancer. To our knowledge, this is the first report of a side-by-side genome-wide comparison of HuR-associated targets in wild type ER+ and ER- breast cancer cells. Our findings indicated that HuR interacts with small subsets of genes in breast cancer, out of the possible 8% of human genes possessing AREs which are potential HuR targets. Three broad categories of HuR targets were identified. First, there was a subset of targets only found in ER+ breast cancer. Second, there was a unique subset of HuR targets found only in ER- breast cancer. A third subset consisted of HuR-associated mRNAs common to both forms of breast cancer, many of which were previously described as having roles in cancer.

We selected and validated two HuR targets, *CD9* and *CALM2* mRNAs, which were found in high abundance in both types of breast cancer. Initially, we employed the previously developed "heat map" signature of HuR binding to

gain insight into putative HuR target sequences [30]. HuR binding was verified by HuR immunoprecipitations, and analyzed by quantitative RT-PCR and biotin pull-downs. Both *CD9* and *CALM2* mRNAs were enriched in HuR RIPs compared to isotype control IP reactions. Biotin pull-downs verified the binding of HuR protein specifically to the 3'UTR regions of both mRNAs, as had been predicted. CD9 is a tetraspanin molecule which plays important roles in cellular development, activation, growth and motility. It has been implicated in a variety of cancers, including but not limited to gastric cancers and B cell acute leukemia [58-60].

The role of CALM2 in cancer is less well understood but may be linked to cancer since it is involved in controlling calcium signaling [61,62]. There are three *CALMODULIN* genes (*CALM1, CALM2* and *CALM3*) highly expressed in both MB-231 and MCF-7 cell lines (Additional File 4, Figure S1). Interestingly, although they are encoded by different genes at different chromosomal locations, all three encode the same open reading frame but differ in the 5' and 3' UTRs [[61,63], and [64]]. Only *CALM2* mRNA interacts with HuR by RIP analysis. Moreover, previously published reports have indicated the necessity of knocking down all three *CALMODULIN* mRNAs by siRNA to achieve knock-down of the protein [61]. We conclude that there may be differential HuR associated regulation of these *CALMODULIN* genes in breast cancer, even though the mechanism needs to be further delineated.

Surprisingly, the regulation of both *CD9* and *CALM2* target genes was dependent upon the cellular milieu. To test the functional consequences of HuR binding to these two transcripts, we prepared cells that stably expressed higher or lower HuR, compared to the parent cells, in both ER+ and ER- breast cancer cell lines. HuR appears to differentially regulate the expression of *CD9* in opposite directions in the two different forms of breast cancer. Specifically, HuR over-expression in ER- breast cancer (MB-231) paradoxically decreased *CD9* mRNA and protein levels, whereas HuR knock-down increased the *CD9* mRNA levels. This is the opposite of what is predicted for most HuR targets, since HuR is thought to stabilize its mRNA targets and often increases their translation. There did not seem to be similar effects upon *CALM2* expression. As expected, knock-down of HuR by shRNA decreased expression of *CD9* and *CALM2* in ER+ breast cancer (MCF-7). Though there are differences in cytoplasmic HuR levels in MB-231 cells as compared with MCF-7, these are modest (10%). This is in keeping, however, with observations that MB-231 cells are more undifferentiated and more aggressive.

Moreover, analysis of HuR-associated mRNAs in both ER+ and ER- breast cancer revealed three broad categories of genes. First, there were well known cancer genes, such as *PTMA*, which are regulated by HuR [27]. Second, there were cancer-related genes, such as *CD9* and *CALMODULIN*, which were not known to be HuR regulated until this report. Third, there were other genes identified by HuR association with unknown cancer function. These could potentially represent novel cancer targets. Additional proof of HuR involvement with other known cancer genes, such as *CD44* and *GATA-3*, may represent novel insights into the mechanisms of regulation of these cancer targets (see Additional Files). These results may therefore advance the field by shedding insights into posttranscriptional regulation of known and perhaps unknown cancer target genes.

Though the exact mechanisms of HuR differential regulation of *CD9* and *CALM2* are presently unclear, it may involve microRNA (miRNA) regulation. In a recent report, we described the recruitment by HuR of miRNA let-7 to translationally silence *C-MYC* expression [46]. It is clear from the findings of laboratories headed by Filipowicz, Steitz and other investigators, that RBPs and miRNAs are involved in intricate associations to affect downstream translational suppression or activation of target mRNAs to help meet cellular needs [65,66]. Sharp and colleagues proposed that different interactions between RBPs and miRNAs may have evolved as a protective mechanism for the cell against environmental stress [67].

A remaining question is why HuR selectively binds to certain genes containing AREs. Our previous work has demonstrated the role that HuR plays in myogenesis by stabilizing the expression of three critical genes involved in myogenesis: *MYOD*, *MYOGENIN*, and $p21^{cip1}$ [68]. HuR over-expression results in precocious muscle differentiation and HuR siRNA knock-down prevents muscle differentiation [69]. It is highly probable that there are more than three HuR targets inside these cells. A specific phenotype potentially arises when HuR levels are altered which may involve interactions with miRNAs, although this theory needs to be fully investigated.

Our findings share some similarity to earlier reports of HuR RIP-Chip analysis of MCF-7 cells stably transfected with MCT-1 [45]. These analyses, however, were not genome-wide and employed transfected cells. Nevertheless, thrombospondin, a known important anti-angiogenic factor, was identified as a HuR-regulated target. Combined with earlier reports of the role of HuR in regulating, VEGF-α and HIF1α, HuR may be controlling a "posttranscriptional mini-operon" involved in angiogenesis [29,32,70]. Further studies are being conducted in our laboratory to investigate the role of HuR in breast cancer angiogenesis using xenograft animal models. It will be particularly important to test the role of HuR upon *CD9* and *CALM2* expression in breast tumors *in vivo*.

Posttranscriptional gene regulation is increasingly being appreciated as a driver of malignant transformation. The roles of both RBPs and miRNAs (so-called oncomirs) are being recognized in cancer [71]. Many reports have described alterations in miRNA expression profile and function as contributing to breast cancer malignant transformation and metastasis [72-75]. HuR RIP-Chip analysis may shed further light into malignant breast cancer transformation by identifying HuR associated mRNAs.

We believe that there are potential applications for tamoxifen resistance as well. Keen and colleagues have described a potential mechanistic link between HuR expression and tamoxifen drug resistance [76]. As breast cancer cells acquire tamoxifen resistance, there are increased levels of cytoplasmic HuR expression. Increased cytoplasmic HuR levels have previously been described in situations where HuR actively influences expression of cytoplasmic targets [18,47,48]. Drug resistance could be reversed by using siRNA to knock-down HuR expression, whereas exogenous over-expression of HuR could cause cells to become resistant to tamoxifen. We therefore propose that HuR may be coordinately regulating genes which may allow a cell to acquire tamoxifen resistance. It will be interesting to further investigate HuR-associated target genes in ER+ cells in this light.

Conclusion

In summary, using RIP-Chip analysis, we have performed for the first time a genome-wide comparison of HuR-associated targets in wild type ER+ and ER- breast cancer. We have identified novel HuR targets and have gained insight into the role HuR plays in regulating known cancer genes. We found distinct, differentially expressed subsets of HuR cancer related genes in ER+ and ER- breast cancer cell lines. Based on our observations, the enhanced expression of these mRNA subsets by HuR can influence many of the acquired capabilities of cancer cells. Further investigation into HuR's role in regulating these genes may provide novel insights into breast cancer diagnosis and therapy.

Appendix

Microarray Data Preprocessing

Data quality was examined by looking at quality controls metrics produced by Illumina's software (BeadStudio v3.1.3.0, Gene Expression Module 3.2.7). The data were then exported for further analyses in R. Image plots of each array were examined for spatial artifacts, and there was no evidence of systematic effects indicative of technical problems with the arrays. Within limma, quantile normalization was used for between chip normalization. Finally, quality control statistics were computed using a variety of Illumina's internal control probes that are replicated on each array. Any probes which were considered "not detectable" across all samples were excluded from further statistical analyses in order to reduce false positives. The determination of "not detectable" was based upon the BeadStudio computed detection p-value being greater than 1%.

Gene Ontology Gene Universe

In defining the gene universe for the analysis, non-specific filtering was used to increase statistical power without biasing the results. We started with all probes on the Illumina array which had both an Entrez gene identifier [77] and a GO annotation, as provided in the lumiHumanAll.db [78] annotation data package and GO.db [79] annotation maps (built using data obtained from NCBI on 4/2/08). This set was then reduced by excluding probes that exhibited little variability (interquartile range (IQR) of <0.1 on $\log_2$ scale) across *all* samples because such probes are generally not informative. Finally, for probes that mapped to the same Entrez identifier, a single probe was chosen in order to insure a surjective map from probe IDs to GO categories (via Entrez identifiers). This was necessary to avoid redundantly counting GO categories which produces false positives. Probes with the largest IQR were chosen to be associated with an Entrez identifier.

Additional file 1: Figure S2. Table of complete GO analysis. Listing of HuR-associated genes with odds ratios and functional categories.

Additional file 2: Table S1. HuR targets five fold or greater. Listing of HuR-associated mRNAs in MB-231 and MCF-7 cell lines.

Additional file 3: Figure S3. Total cellular levels of HuR are similar in MB-231 and MCF-7 cells. Nuclear and cytoplasmic separation was performed to measure levels of HuR in different compartments of MB-231 and MCF-7 cells. Total cellular HuR levels were very similar, whereas there was a small (10%) increase in HuR cytoplasmic levels in MB-231 cells as compared to MCF-7. Absence of β-tubulin staining demonstrates integrity of isolation as there should not be β-tubulin in the nuclear fraction. Bands were measured by densitometry and normalized to β-tubulin controls. (T = total cellular lysate; C = cytoplasmic lysate, N = nuclear lysate).

Additional file 4: Figure S1. Relative baseline values of *CALM1*, *CALM2*, *CALM3*, and *CD-9* mRNAs in ER+ and ER- cells. Quantitative RT-PCR performed on mRNA extracted from cell lysates showing relative levels of *CALM1*, *CALM2*, *CALM3*, and *CD-9* mRNAs in MB-231 and MCF-7 breast cancer cells. All values were normalized to *GAPDH* mRNA. All experiments were done in triplicate (n = 3) except for *CALM3* (n = 2).

Abbreviations

(ER-): Estrogen receptor negative; (ER+): estrogen receptor positive; (RIP): RNA immunoprecipitation; (RIP-Chip): RNA immunoprecipitation applied to microarrays; (3' UTR): 3' untranslated region; *ELAV1*: *(embryonic lethal abnormal vision 1)*.

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Author details
[1]Departments of Surgery, University of Missouri, One Hospital Drive, Columbia, Missouri 65212, USA. [2]Health Management and Informatics, University of Missouri, 187 Galena Hall, Columbia, Missouri 65212, USA. [3]Statistics, University of Missouri, 146 Middlebush Hall, Columbia, Missouri 65211, USA. [4]Molecular Microbiology and Immunology, One Hospital Drive, Columbia, Missouri 65212, USA. [5]Child Health, One Hospital Drive, University of Missouri, Columbia, Missouri 65212, USA. [6]National Institute of Aging, 251 Bayview Boulevard, Baltimore, Maryland 21224, USA.

Authors' contributions
UA supervised the study. RC planned the experiments, performed microarrays and wrote the paper. MMG generated and performed analyses on cell lines as well as quantitative RT-PCR experiments. JWD performed statistical analyses on microarray experiments. JDM assisted in analyses of cell lines. YK and MG planned and performed biotin pull-down experiments. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Overexpression of the RNA binding protein HuR impairs tumor growth in triple negative breast cancer associated with deficient angiogenesis

Matthew M. Gubin,[1,2] Robert Calaluce,[1] J. Wade Davis,[7,8] Joseph D. Magee,[1] Connie S. Strouse,[2] Daniel P. Shaw,[6] Lixin Ma,[9] Ashley Brown,[9] Timothy Hoffman,[4,5,9] Tammy L. Rold[4,9] and Ulus Atasoy[1-3,*]

[1]Departments of Surgery; [2]Molecular Microbiology and Immunology and [3]Child Health; [4]Internal Medicine and [5]Chemistry; [6]Veterinary Pathobiology; [7]Health Management and Informatics; [8]Statistics; [9]Harry S. Truman VA Hospital; University of Missouri; Columbia, MO USA

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Abbreviations: ER$^-$, estrogen receptor negative; ER$^+$, estrogen receptor positive; elav, embryonic abnormal vision; RBP, RNA-binding proteins; RIP, RNA immunoprecipitation; miRNA, microRNA

Interactions between RNA binding proteins (RBPs) and genes are not well understood, especially in regulation of angiogenesis. The RBP HuR binds to the AU-rich (ARE) regions of labile mRNAs, facilitating their translation into protein and has been hypothesized to be a tumor-maintenance gene. Elevated levels of cytoplasmic HuR directly correlate with increased invasiveness and poor prognosis for many cancers, including those of the breast. HuR controls the expression of multiple genes involved in angiogenesis including VEGFα, HIF1α and thrombospondin 1 (TSP1). We investigated the role of HuR in estrogen receptor negative (ER$^-$) breast cancer. MDA-MB-231 cells with higher levels of HuR have alterations in cell cycle kinetics and faster growth. Unexpectedly, HuR overexpression significantly interfered with tumor growth in orthotopic mouse models. The putative mechanism seems to be an anti-angiogenetic effect by increasing expression of TSP1 but also surprisingly, downregulating VEGF, a target which HuR normally increases. Our findings reveal that HuR may be regulating a cluster of genes involved in blood vessel formation which controls tumor angiogenesis. An approach of modulating HuR levels may overcome limitations associated with monotherapies targeting tumor vessel formation.

Introduction

Although most breast cancers are estrogen receptor positive (ER$^+$), approximately 15% are estrogen receptor negative (ER$^-$).[1] In general, ER$^-$ tumors are more aggressive, have a poor clinical outcome and dismal survival rates that disproportionately affect lower income and minority women. The poor prognosis is partly attributed to the fact that these women are limited to conventional cytotoxic chemotherapy. This is in distinct contrast to ER$^+$ tumors, which can be treated with hormonal therapies such as tamoxifen or aromatase inhibitors. Moreover, the presence of the growth factor HER2 allows oncologists to employ targeted therapy using HER2 receptor-specific drugs.

The role of posttranscriptional gene regulation in cancer is now widely appreciated, as the contributions of RNA binding proteins (RBPs) and microRNAs (miRNAs) are becoming more apparent.[2-8] The posttranscriptional operon hypothesis states that RBPs and miRNAs are coordinately regulating expression of biologically related mRNAs. This has generated interest in identification of additional gene products in the acquired capabilities model of malignant transformation.[9] The RBP HuR, a paraneoplastic antigen, overexpressed in many malignancies including breast cancer, has been implicated as an important RBP which may function as a tumor maintenance gene, facilitating malignant transformation.[10-17] HuR has been shown to regulate genes in multiple areas of the acquired capabilities model, including two pivotal genes involved in angiogenesis, VEGF and HIF1α.[18-22] Increased HuR cytoplasmic expression directly correlates with severity and aggressiveness of many cancers, including those of the breast.[23-25] HuR has been demonstrated to be an important prognostic factor in familial breast cancer patients.[23,24] Recently, investigators altered the subcellular distribution of HuR and documented a decrease in mRNA stability, subsequent ER levels and susceptibility to tamoxifen.[26]

In this study we investigated the role of HuR in ER$^-$ breast cancer by gain-of-function ER$^-$ (MDA-MB-231) cell lines which overexpressed epitope tagged hemagglutinin (HA) HuR. As expected and consistent with published results for other cancer types, HuR overexpression in vitro resulted in increasing MDA-MB-231 cellular growth rates and alterations in cell cycle

*Correspondence to: Ulus Atasoy; Email: atasoyu@missouri.edu
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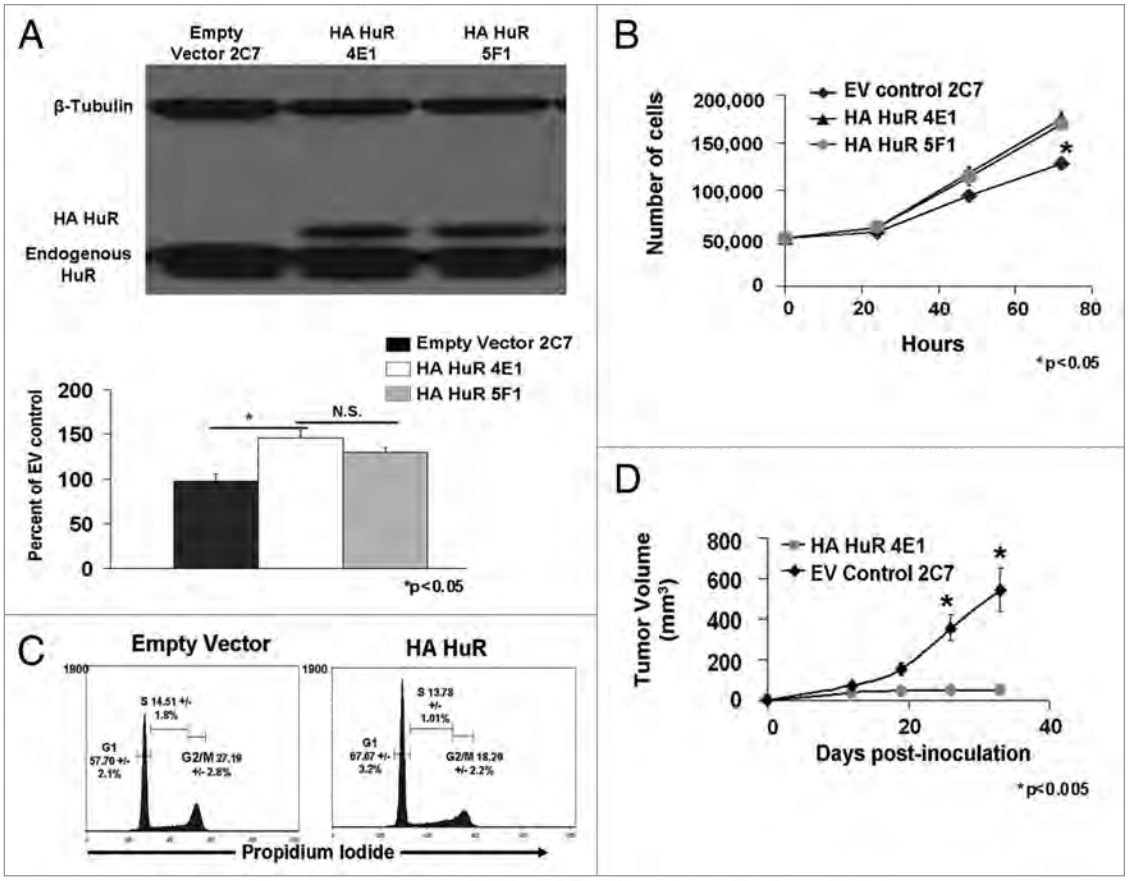

Figure 1. Overexpression of HA-HuR in MDA-MB-231 cancer cells increases cell growth and alters cell cycle kinetics in vitro while inhibiting tumor growth in vivo. (A) MDA-MB-231 cells transfected with the pZeoSV2 vector expressing HA-HuR, selected with Zeocin and cloned by limiting dilution express HA-HuR compared to a pZeoSV2 empty vector control. MDA-MB-231 clones 4E1 and 5F1 expressed 42% and 38% more HuR than control as shown in representative western blot. Bar graph represents mean HuR percent overexpression from three different western blots. (B) Both clones expressing HA-HuR proliferated significantly faster than the empty vector control in vitro. (C) Overexpression of HA-HuR increased cells in G_0/G_1 cell cycle, from 57.70% to 67.67%. Overexpression of HA-HuR decreased cells in G_2/M phase by 27.19% to 18.29% but did not significantly alter cells in S phase as measured by DNA content. (D) MDA-MB-231 HA-HuR 4E1 showed significantly reduced tumor volume (mm^3) and growth starting at two weeks post-inoculation and continuing for five weeks when compared to empty vector MDA-MB-231 controls as measured by both MRI and calipers. For tumor experiments nine animals per group were used. For both counting assay and tumor volumes data represent mean value ± SEM. $p < 0.05$. Flow cytometry data represent mean value ± SEM from n = 4 separate experiments done in triplicate. $p < 0.05$.

kinetics. Surprisingly, when these cells were used in vivo as xenografts in athymic mice, a 90% reduction in growth rate was seen, as compared to control groups. The presumptive mechanism was an anti-angiogenic affect by increasing the expression of thrombospondin 1 (TSP1), a gene known to be anti-angiogenic. However, unexpectedly, there were also decreases in VEGF expression. Thus, our results indicate that HuR overexpression in ER$^-$ breast cancer can potently inhibit tumor growth by potentially blocking angiogenesis.

Results

Overexpression of HuR in MDA-MB-231 cells increases growth rates and alters cell cycle kinetics. To study the role of HuR expression in MDA-MB-231 ER$^-$ breast cancer we made individual clones which overexpressed either epitope tagged (HA) HuR or empty vector (EV) control and measured growth rates and cell cycle kinetics (**Fig. 1A**). Overexpression of HA-HuR resulted in accelerated cellular growth as determined by counting (**Fig. 1B**). When the cells were stained with propidium iodide, we noted an alteration in cell cycle kinetics (**Fig. 1C**). HA-HuR overexpressing cells had increased amounts of cells in G_1 (67 vs. 57%), as compared with empty vector controls. Conversely, HuR overexpression also resulted in a compensatory decrease in G_2/M percentages (18% vs. 27%). We concluded, as expected, that HuR overexpression resulted in increases in growth rates of MDA-MB-231 cells. We then investigated the effects of HuR overexpression in vivo, using orthotopic xenograft animal models.

HuR overexpression results in significantly reduced tumor growth and mass. The clones used in **Figure 1**, empty vector (2C7) and overexpresser HA-HuR (4E1), were injected into the contralateral mammary fat pads of athymic nude mice. Tumor growth was assessed weekly by caliper measurements and followed in vivo by MRI scan. Surprisingly, tumors overexpressing HuR had significant inhibition of growth, whereas EV control tumors increased significantly in tumor volume (**Fig. 1D**). The

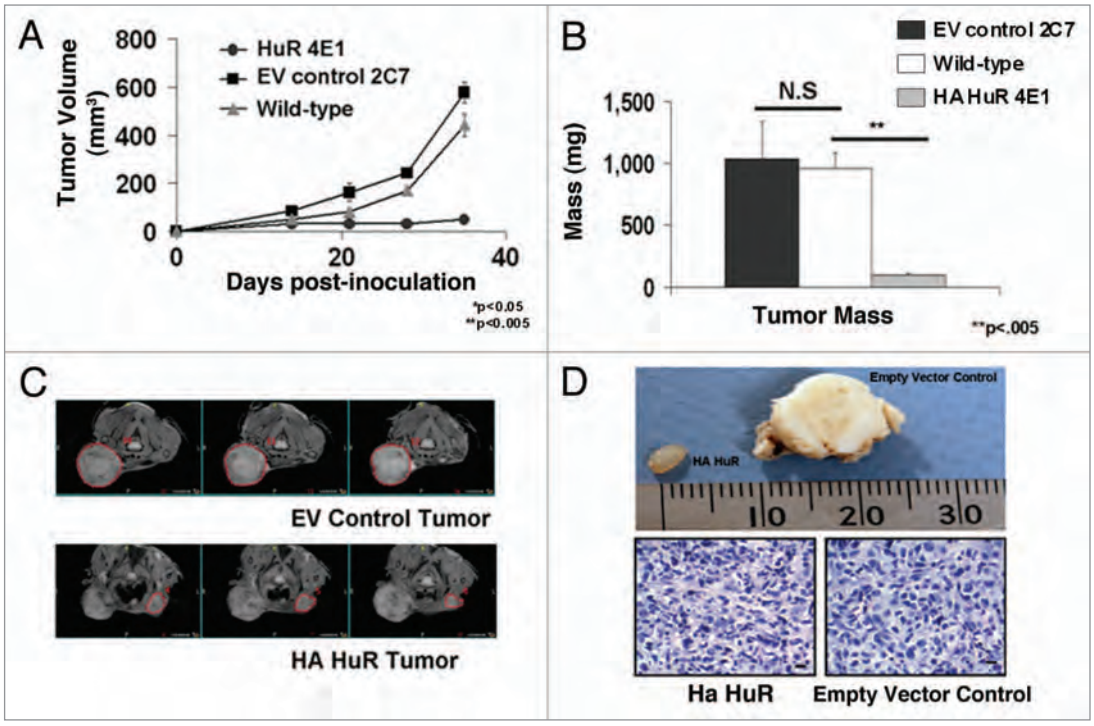

Figure 2. Overexpression of HA-HuR in MDA-MB-231 cancer cells inhibits tumor growth in athymic nude mice. (A) Repeat experiments comparing MDA-MB-231 HA-HuR 4E1 with both wild-type MDA-MB-231 and empty vector MDA-MB-231 confirmed HuR overexpression reduced tumor volume (mm^3) and growth starting at two weeks post-inoculation and continuing for five weeks as measured by calipers. (B) Tumors overexpressing HA-HuR had significantly less mass after harvest 42 days post-inoculation when compared to the WT or empty vector (EV) controls. (C) MRI comparing largest sections for each tumor showed significantly smaller tumors in the HA-HuR overexpressing tumors when compared to EV control tumors. (D) Representative cross sections of tumors showed that those formed by inoculation with HA-HuR resembled a gelatin-like capsule, were significantly smaller and more homogeneous than those formed by inoculation with empty vector. Hematoxylin and eosin stain revealed poorly differentiated carcinomas with similar morphology and lack of inflammatory cells in both HA-HuR tumors and EV control tumors. Five animals were used per group in HA-HuR, empty vector and wild-type control groups. Experiments were repeated with similar results using a different clone, 5F1, (see Supporting Information **Fig. S3**). Data represent mean value ± SEM. $p < 0.05$; in photomicrographs bar = 27 microns.

tumors were removed from the animals on day 42. Histological staining, western blotting and RT-PCR were performed to determine whether any cells remained. As seen in **Supplementary Data**, both control (2C7) and HuR (4E1) tumors had intact human GAPDH mRNA and tumor 4E1 expressed the HA-HuR transgene. Furthermore, the HA-HuR tumors harvested on day 42 still expressed HA-HuR protein (**Fig. S1**). We reestablished cell lines from tumors removed from animals to study their growth rates. We found that these reestablished cells had similar growth rates to parental cells prior to transplantation (**Fig. S2**).

The experiments were repeated with wild-type, parental MDA-MB-231 cells to validate these findings. As seen in **Figure 2**, both parental and control cells (2C7) grew at similar rates, whereas tumors which overexpressed HuR (4E1) did not appear to grow (**Fig. 2A**). Tumor mass was assessed and showed that HuR overexpression resulted in a 90% reduction in growth (**Fig. 2B**). These results were confirmed by MRI scans, gross photographs and microscopy (**Fig. 2C and D**). HA-HuR tumors appeared to be a gelatin-like capsule and the control tumors were a solid round mass. Cross sections of both revealed that the HA-HuR tumors had a smooth, homogeneous and glistening surface, whereas the control tumors had a heterogeneous, yellow-white surface with a necrotic center (**Fig. 2D**). Both tumors contained viable cancer cells and similar morphology determined to be moderately to poorly differentiated carcinoma, consistent with the implanted MDA-MB-231 cells (**Fig. 2D**). We concluded that HuR overexpression resulted in significantly smaller ER^- tumors in animals.

To verify that these results were not clonal, we repeated the orthotopic tumor injection experiments using a second HuR overexpressing clone (5F1) which in vitro grew similarly to the original overexpressing clone, 4E1 (**Fig. 1B**). As seen in **Figure S3**, tumor 5F1 also exhibited retarded growth rates as compared to empty vector controls and had a 90% reduction in mass (**Fig. S3**). Taken together, these findings demonstrated that HuR overexpression in MDA-MB-231 cells resulted in significant reductions in tumor growth in a clonal independent fashion.

Gene ontology (GO) analysis of overexpressed genes in HuR overexpressing cells. In order to better understand the genes which may be involved in altering tumor growth in HuR overexpressing MDA-MB-231 cells, we performed genome wide microarray analysis (**Fig. S4 and Table 1**). As seen in **Figure S4**, many genes were over-represented (reflected in large odds ratios) that deal with both biological processes as well as molecular function. Given the large numbers of genes, we decided to investigate the following three potential mechanisms to explain

Table 1. Genes upregulated in tumors overexpressing HuR

Fold change	Entrez ID	Gene name
21.52	79191	IRX3/iroquois homeobox 3
16.87	4256	MGP/matrix Gla protein
12.21	1278	COL1A2/collagen, type I, alpha2
9.35	8714	ABCC3/ATP-binding cassette, sub-family C (CFTR/MRP), member 3
7.58	221476	PI16/peptidase inhibitor 16
7.46	972	CD74/CD74 molecule, major histocompatibility complex, class II invariant chain
6.90	2202	EFEMP1/EGF-containing fibulin-like extracellular matrix protein 1
6.84	5265	SERPINA1/serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
6.77	3399	ID3/inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
6.63	6275	S100A4/S100 calcium binding protein A4
5.85	57537	SORCS2/sortilin-related VPS10 domain containing receptor 2
5.07	6275	S100A4
5.07	1831	TSC22D3/TSC22 domain family, member 3
4.87	51313	C4orf18/chromosome 4 open reading frame 18
4.87	4061	LY6E/lymphocyte antigen 6 complex, locus E
4.77	3108	HLA-DMA/major histocompatibility complex, class II, DM alpha
4.61	10507	SEMA4D/sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D
4.46	7052	TGM2/transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)
4.46	1728	NQO1/NAD(P)H dehydrogenase, quinone 1
4.43	9022	CLIC3/chloride intracellular channel 3
4.42	7057	THBS1/thrombospondin 1
4.36	7857	SCG2/secretogranin II (chromogranin C)
4.29	4599	MX1/myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
4.24	22998	LIMCH1/LIM and calponin homology domains 1
4.14	3371	TNC/tenascin C
4.13	6376	CX3CL1/chemokine (C-X3-C motif) ligand 1
4.1	199	AIF1/allograft inflammatory factor 1
4.05	652	BMP4/bone morphogenetic protein 4
3.94	7058	THBS2/thrombospondin 2
3.86	26227	PHGDH/phosphoglycerate dehydrogenase
3.78	144165	PRICKLE1/prickle homolog 1 (Drosophila)
3.76	4147	MATN2/matrilin 2
3.75	8140	SLC7A5/solute carrier family 7 (cationic amino acid transporter, y^+ system), member 5
3.54	8460	TPST1/tyrosylprotein sulfotransferase 1
3.48	64764	CREB3L2/cAMP responsive element binding protein 3-like 2
3.44	3397	ID1/inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
3.42	9516	LITAF/lipopolysaccharide-induced TNF factor
3.4	55971	BAIAP2L1/BAI1-associated protein 2-like 1
3.36	80063	ATF7IP2/activating transcription factor 7 interacting protein 2
3.26	1021	CDK6/cyclin-dependent kinase 6
3.25	7100	TLR5/toll-like receptor 5
3.17	94121	SYTL4/synaptotagmin-like 4
3.16	8622	PDE8B/phosphodiesterase 8B
3.15	55616	DDEFL1/ArfGAP with SH3 domain, ankyrin repeat and PH domain 3

Tumor microarray reveals 47 annotated genes up regulated in the HA-HuR overexpressing tumors as compared to EV control tumors. 47 genes are up regulated 3-fold or greater in the HA-HuR tumors as compared to EV control tumors (false discovery rate <1%), and also have a probability of differential expression >80% based on a Bayesian analysis.

Table 1. Genes upregulated in tumors overexpressing HuR (continued)

3.13	30818	KCNIP3/Kv channel interacting protein 3, calsenilin
3.13	3430	IFI35/interferon-induced protein 35
3.07	29887	SNX10/sorting nexin 10
3.01	23052	ENDOD1/endonuclease domain containing 1

Tumor microarray reveals 47 annotated genes up regulated in the HA-HuR overexpressing tumors as compared to EV control tumors. 47 genes are up regulated 3-fold or greater in the HA-HuR tumors as compared to EV control tumors (false discovery rate <1%), and also have a probability of differential expression >80% based on a Bayesian analysis.

the large discrepancy seen in tumor growth: (1) increased apoptosis, (2) increases in senescence and (3) alteration in angiogenesis. The microarray profiling, however, did not distinguish between direct and indirect HuR targets. Therefore, we decided to further investigate known HuR targets related to cancer.

Tumors which overexpress HuR have decreased angiogenesis. We conducted experiments which targeted well known HuR target genes involved in angiogenesis which had previously been described in the literature: thrombospondin 1 (TSP1), VEGF and HIF1α.[21,22,27] We therefore performed real-time PCR to measure mRNA levels of TSP1, VEGF and HIF1α. As seen in **Figure 3A**, HuR overexpression caused an increase in TSP1 mRNA and protein (**Fig. 3B**) but surprisingly decreases in steady-state VEGF mRNA and protein levels; HIF1α steady-state mRNA levels appeared unchanged (**Fig. 3A–C**). We further measured HIF1α protein by western and immunohistochemical staining, which were unchanged between HuR overexpressing and control tumors (data not shown). Furthermore, RNA immunoprecipitation (RIP) using an antibody specific to HuR and an IgG1 antibody control revealed that HuR interacts with TSP1 and VEGF mRNA, as these transcripts were 38- and 54-fold enriched, respectively, in the HuR IP pellet, as compared with istotype control. TSP1 and VEGF mRNA were upregulated 62- and 59-fold in the HuR IP from the HuR overexpression cells. GAPDH, which does not interact with HuR showed no enrichment in either HuR IP when compared to the IgG1 IP (**Fig. 4A**). After confirming HuR interacts with TSP1 and VEGF mRNA we sought to gain a better understanding of the mechanism by which the overexpression of HuR leads to increases in TSP1 mRNA and protein and decreases in VEGF mRNA and protein. To determine whether alterations in HuR levels were altering TSP1 and VEGF mRNA stability we performed mRNA decay assays using actinomycin D. The VEGF mRNA stability was not altered when comparing HuR overexpressing cells with empty vector control (**Fig. 4B**). TSP1 mRNA half-life was increased in the HuR overexpressing cells when compared to the empty vector control cells (**Fig. 4C**).

We next examined whether alterations in apoptosis could account for differences in tumor growth. To determine whether HuR overexpression was altering apoptosis in vitro we performed both annexin V and 7-AAD staining. No differences in amount of cells undergoing apoptosis between the HuR overexpressers and EV controls were seen (**Figs. 5A and S5**). In vivo, there were no increases in apoptosis in HuR overexpression tumors, as compared with EV control tumors harvested on day 42 post-inoculation (**Figs. 5B and S6**). Increased apoptosis in EV tumors was mostly found in the necrotic centers. To account for the possibility that apoptosis was occurring early in tumor formation, we repeated the experiments and harvested tumors from mice 14 days post-inoculation. No increases in apoptosis were seen in HA-HuR tumors as compared to EV control tumors (**Figs. 5B and S6**). The tumors from mice 14 days post-inoculation were only beginning to be visible. Hematoxylin and eosin staining revealed tumor morphology consistent with breast adenocarcinoma (**Fig. S7**). We further quantitated the amount of blood vessels and found a significant decrease in vessel formation in HA-HuR tumors as compared with EV controls (**Fig. 5B and C**). No tortuous vessels were seen. These non-functional vessels are sometimes seen when there are perturbations in the DLL4-Notch signaling pathways. Measurement of senescence using β-galactosidase staining did not reveal any significant differences (**Fig. S8**). Taken together, we concluded that HuR overexpression in MDA-MB-231 tumors does not appear to alter cellular apoptosis but may interfere with angiogenesis by overexpression of an anti-angiogenetic factor, TSP1 and by downregulation of a known pro-angiogenic factor, VEGF.

Discussion

In ER^- cells overexpressing HuR, there are increases in growth rates in vitro as well as alterations in cell cycle kinetics. Specifically, MDA-MB-231 cells overexpressing HuR have increases in the G_1 phase of the cell cycle, consistent with published results.[25] A plausible explanation is HuR induced stabilization of cyclin B1, the pivotal cyclin involved in G_2/M transition.[28] Surprisingly, when these same cells were transplanted into athymic nude mice, HuR overexpression resulted in a 90% growth reduction in tumor size. These results were confirmed by measuring tumor volume and mass. Additionally, the results were validated by serial MRI scans during the experimental period and further confirmed in two independent clones. Empty vector (EV) control and parental wild type MDA-MB-231 cells grew similarly and resulted in much larger tumors than those formed by HA-HuR overexpressing cells. Histological staining of cross sections from EV and HA-HuR tumors revealed them to be poorly differentiated carcinomas, consistent with ER^- breast cancer. Moreover, the HuR transgene was still expressed in the smaller tumors at the time of harvest (day 42).

We searched for putative mechanisms to explain these findings, since in other systems HuR overexpression results in larger, more robust tumor growth.[25] No inflammatory infiltrates were

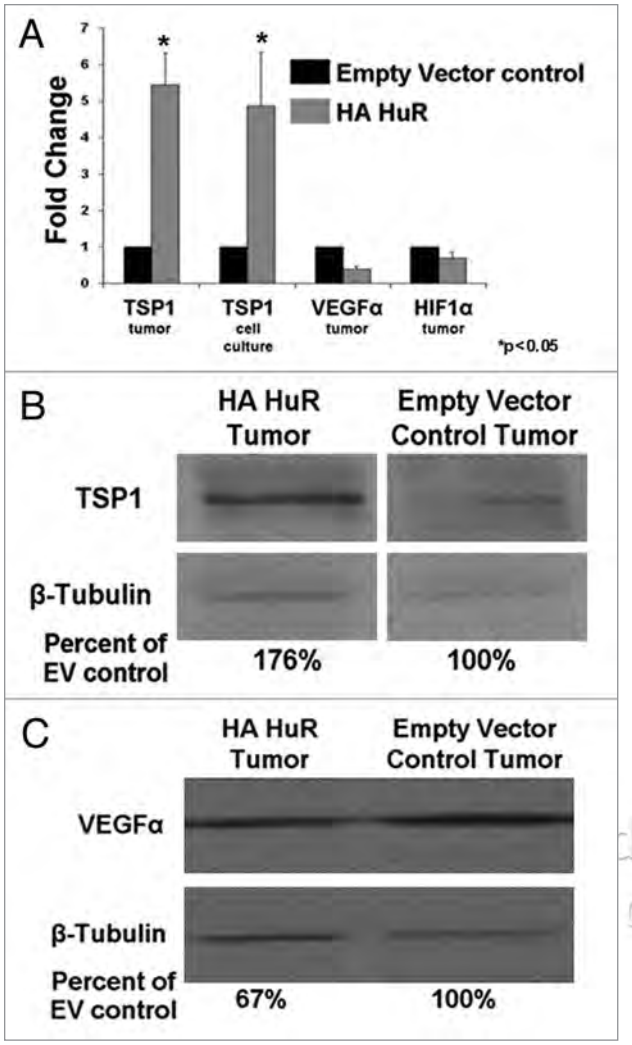

Figure 3. TSP1 is upregulated in HA-HuR tumor and VEGFα is downregulated. (A) Real-time PCR indicates TSP1 is upregulated in tumors (5.44 fold) and cells in culture (4.88 fold) overexpressing HA-HuR when compared to EV control tumors and cells, consistent with the microarray data. VEGF is downregulated (2.6 fold) in tumors overexpressing HA-HuR when compared to EV controls. HIF1α mRNA levels did not appreciably change. Change in gene expression was determined using the comparative C_T method and is represented as fold change in HA-HuR tumors as compared to empty vector controls. GAPDH was used as an endogenous control. (B) Western blot for TSP1 shows increased protein expression of TSP1 (76%) in the HA-HuR overexpressing tumors when compared to EV control tumors. (C) Western blot for VEGF shows decrease protein expression by 23% in the HA-HuR overexpressing tumors when compared to EV control tumors (representative of two independent sets of tumors). Data represent mean value ± SEM from n = 3 separate mice done in triplicate. $p < 0.05$.

seen in either tumor. As expected, there was increased apoptosis in the centers of the EV control tumors, since these regions are relatively more hypoxic. Apoptosis early in tumor development is an unlikely mechanism of inhibited tumor growth in the tumors overexpressing HuR as we did not see any alterations in apoptosis 14 days post-inoculation using two methods of apoptosis detection. Furthermore, significant apoptosis was not observed in either cell lines (in vitro) or tumors (in vivo) overexpressing HuR. These results more than likely rule out apoptosis as a mechanism of reduced tumor growth in HA-HuR tumors, although we cannot definitely exclude apoptosis earlier than 14 days. Since the tumors continue to overexpress HuR even at day 42, it would seem unlikely that HuR overexpression would be the etiology of cell death. We also ruled out senescence as a mechanism, as there was less β-galactosidase staining in the smaller tumors.

Due to previously published data on the role of HuR in controlling angiogenesis via interactions with VEGF and HIF1α mRNAs, we investigated the relationship between HuR overexpression and these pro-angiogenetic factors.[21,22] There was a statistically significant decrease in VEGF mRNA and protein expression and no increases in HIF1α mRNA or protein expression. As expected, increased HuR expression correlated with increased TSP1 mRNA expression and protein levels. TSP1 is a well known anti-angiogenetic factor and has been described to be regulated by HuR.[27] Enumeration of angiogenesis by CD34 staining confirmed that HuR overexpression resulted in significant decreases in new blood vessel formation. This decrease in neovascularization may, in part, explain why tumors formed by HA-HuR overexpression were much smaller than EV controls.

Other groups have shown that decreases in VEGF expression in a variety of cancer models can inhibit angiogenesis and tumor growth.[29-32] It has also been demonstrated that TSP1 can mediate tumor growth suppression via blocking angiogenesis, consistent with our results.[33,34]

As previously mentioned HuR has been described to stabilize TSP1 and VEGF mRNA resulting in greater levels and increased protein expression.[21,27] In contradistinction to published reports of HuR stabilization of VEGF mRNA, our findings indicate that under conditions of HuR overexpression, there are decreases in both VEGF mRNA and protein levels. The reasons for this discrepancy are presently unknown, though the results may be dependent upon levels of HuR overexpression and hypoxia. HuR's relationship, however, with HIF1α is more complex. HuR binds to AU-rich regions in HIF1α 5'UTR, rather than the 3'UTR (even though both regions of the molecule possess AREs), and causes a translational upregulation in HIF1α protein synthesis without altering mRNA levels. It appears HuR overexpression is not affecting HIF1α protein production in our system. HIF1α is the major transcriptional factor for VEGF mRNA transcription, and as such, this is an area of intensive investigation in our laboratory.

The exact mechanisms of HuR-induced anti-angiogenetic effects are not completely understood but may involve interactions between HuR and microRNAs. In a recently published report, HuR actively recruited let-7 miRNA to c-myc mRNA, which resulted in decreases of its translation.[35] Furthermore, we do not have any direct evidence that the HA tag (located at the amino terminal) affects the distribution or targeting of HuR to its mRNA targets. Published results from the Kontoyonnis lab used the same epitope tag to make a transgenic mouse overexpressing HA-HuR in macrophages and although this is a different system than ours, they did not see any alterations in nuclear vs. cytoplasmic HuR distribution nor binding to its mRNA

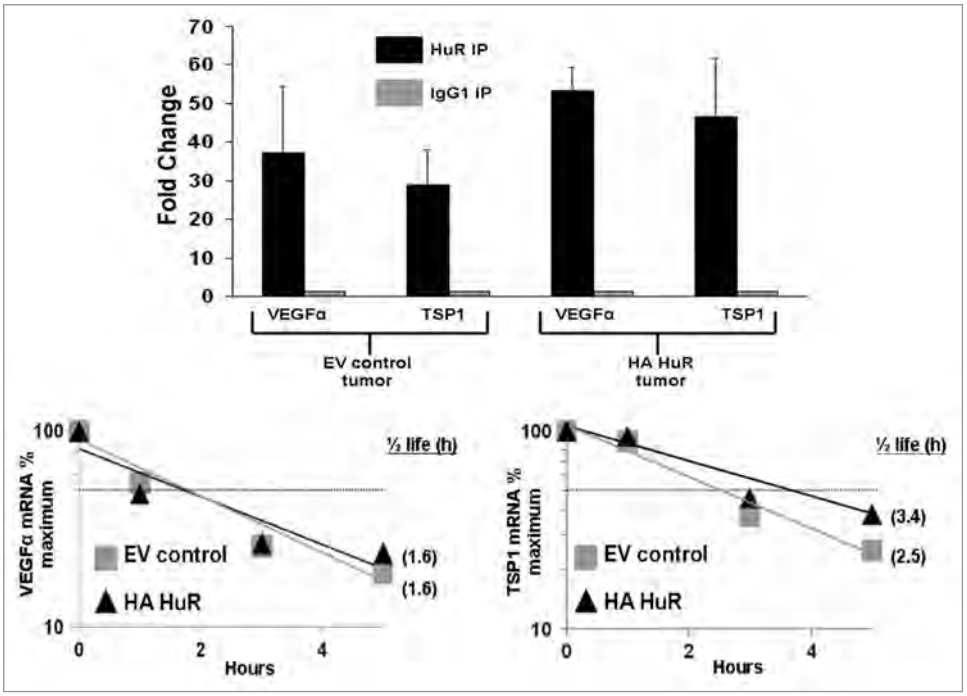

Figure 4. HuR interacts with both TSP1 and VEGF mRNAs in cells overexpressing HuR. (A) RNA immunoprecipiation indicates both TSP1 and VEGF mRNA are increased in the HuR IP when compared to IgG1 control IP in both HA-HuR overexpressing cells and EV control cells. (B) Actinomycin D mRNA stability assay shows VEGF mRNA half-life was not altered between cells overexpressing HA-HuR and EV control cells. (C) TSP1 mRNA from cells overexpressing HA-HuR has a longer half-life than TSP1 mRNA from EV control cells. For RNA immunoprecipitation, data represents mean value ± SEM. $p < 0.05$.

targets.[36] Moreover, the metastatic potential of anti-angiogenetic MDA-MB-231 HuR altered cell lines needs to be investigated, since this phenotype results in patient mortality and the correlation between tumor size and metastatic potential may not be direct. We have identified putative markers of metastasis which have been altered in vivo in these cells and this area is currently under investigation.

Interactions between RBPs such as HuR and their downstream mRNA targets are complex. Even though HuR can theoretically bind 8% of human mRNAs which contain AREs, it does not necessarily do so.[37-39] Published reports from our lab and others demonstrate that HuR critically regulates myogenesis by controlling the expression of three central genes in this process.[40,41] HuR siRNA knock down prevents muscle formation, whereas HuR overexpression results in precocious differentiation.[40,41] Yet, it is highly likely HuR is interacting with many more targets inside these cells. The exact explanations for this discrete phenotype are not presently understood. Our work involving targets discovered by RIP-Chip in cell lines under normoxic conditions indicates that HuR may indeed regulate the same targets in opposite fashion, dependent upon cellular milieu, though the reasons are not presently clear.[42] There are obvious differences when performing these experiments under the hypoxic conditions which operate in animals in which tumors form. The published data has both indicated that HuR overexpression correlates with increased as well as decreased aggressiveness in breast cancer.[23,24,43] These results would lend credence to the idea that HuR may be directing its mRNA targets in cell-specific fashion.

In summary, our findings indicate that HuR overexpression in ER$^-$ breast cancer results in a concomitant increase in TSP1 and a decrease in VEGF expression as well as substantial decreases in tumor size. Presently, the reasons for decreases in VEGF expression in HuR-overexpressing tumors are unclear. Our results are highly reproducible when each mouse is compared with its cohorts within individual and duplicate experiments. Furthermore, it is clone-independent, in that we obtained similar results using two different overexpression clones. However, we do not yet know if these results are specific to only ER$^-$ breast cancer, or also include the ER$^+$ subtype, as our results deal primarily with one cell line and must be validated in other cell lines before we can make generalized conclusions.

It will be interesting to test the therapeutic paradigm whereby exogenous HuR overexpression in already established tumors may abrogate their development, perhaps by interfering with angiogenesis. If correct, HuR may be involved in regulating a cluster of genes involved in angiogenesis and therefore, alterations in HuR expression may influence multiple downstream genes which operate in blood vessel formation. These experiments are currently underway. There has been a great deal of interest in blocking angiogenesis, either by increasing expression of anti-angiogenetic factors, such as TSP1 or blocking pro-angiogenetic factors such as VEGF. It has been challenging to work with TSP1 due to its large molecular size. The emerging evidence in cancer treatment approaches in this area, however, has been that monotherapies can be more readily overcome by cancer cells, which are constantly evolving in the patient. We may have discovered a novel way of limiting ER$^-$ tumor growth by interfering with neo-angiogenesis at multiple steps by modulating the expression of a key RBP involved in tumor growth.

Materials and Methods

Cell line and growth conditions. The MDA-MB-231 cell line was purchased from American Type Culture Collection (Manassas, VA) and grown according to the vendor's directions.

Generation of clones expressing HA-HuR. Hemagglutinin (HA) tagged human HuR was cloned into the NheI and XhoI sites of the pZeoSV2 (-) (Invitrogen™) vector. Cells were then plated and transfected with either pZeo HA-HuR or pZeo empty vector using Lipofectamine 2000 (Invitrogen™).

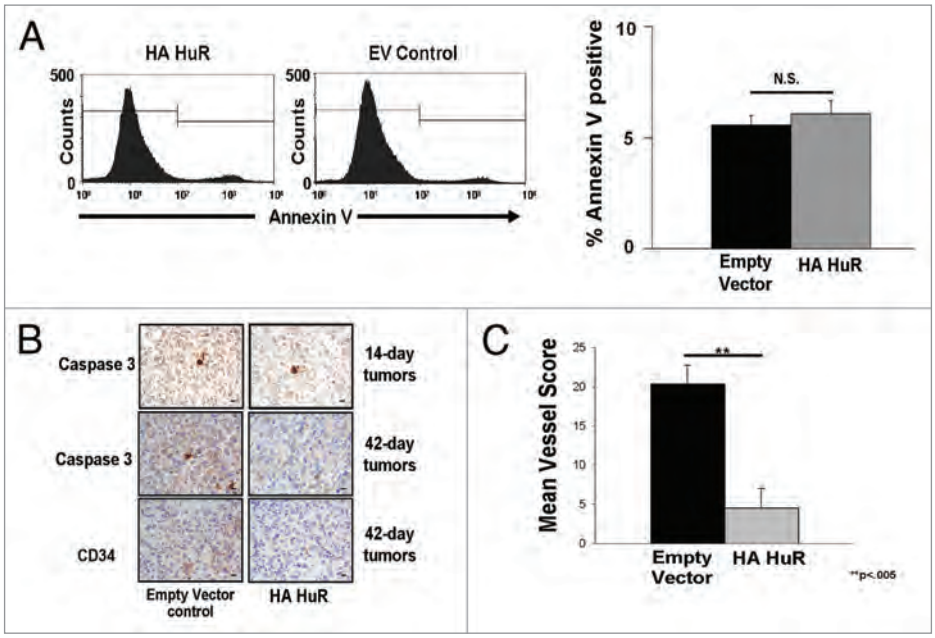

Figure 5. Tumors overexpressing HA-HuR have no increases in apoptosis but decreased blood vessel formation compared to control. (A) Annexin V staining reveals similar amounts of cells undergoing apoptosis between cells overexpressing HA-HuR and EV control cells. (B) Caspase 3 staining shows no differences in the amount of apoptosis in the EV control tumors compared to HA-HuR tumors harvested 14 days post-inoculation. In the tumors harvested on day 42 post-inoculation, caspase 3 staining showed more apoptotic cells in the EV control tumors compared to tumors overexpressing HA-HuR. CD34 staining shows fewer blood vessels in tumors overexpressing HA-HuR. (B) Quantitation of blood vessels stained (number of vessels per high power field scored) with CD34 indicated significantly fewer blood vessels in the tumors overexpressing HA-HuR. Error bars ± SEM; $p < 0.005$; in photomicrographs bar = 27 microns. Representative of n = 5 sets of tumors.

Selection was performed with 200 μg/ml of Zeocin antibiotic (Invitrogen™) and cells were cloned by limiting dilution.

SDS-PAGE and western blot analysis. Western analysis was performed as described previously.[12] For detection of VEGFα and TSP1 from tumors, triple-detergent RIPA lysis buffer with protease inhibitors was used. Membranes were probed with anti-TSP1 (Abcam) or anti-VEGFα (Abcam) and anti-β-tubulin (Sigma-Aldri+ch). Secondary antibodies used were: sheep anti-mouse HRP (GE Healthcare) or donkey anti-rabbit HRP (GE Healthcare) for VEGFα and TSP1, respectively. Proteins were detected using chemiluminescence (GE Healthcare). HuR, TSP1 and VEGFα levels were determined using Bio-Rad's Quantity One software (Bio-Rad) normalizing to β-tubulin. Anti-HuR 3A2 hybridoma was kindly provided by Joan Steitz (Yale School of Medicine).

In vitro growth, apoptosis and cell cycle assay. Fifty thousand cells were seeded in a 24-well plate. Cells were then counted using a hemocytometer and trypan blue exclusion dye 24, 48 and 72 hours post-seeding n = 4 assays. For cell cycle analysis, cells were fixed and permeabilized, re-suspended in PBS with 0.2 mg/ml RNase A (Sigma-Aldrich) and 10 mg/ml propidium iodide (Sigma-Aldrich), and analyzed on FACScan (BD Biosciences, San Jose, CA) and cell cycle analysis was performed using Cell Quest software (BD Biosciences). Annexin V and 7-AAD staining was performed using the FITC Annexin V Apoptosis Detection Kit I (BD Bioscience) by following the manufacturer's protocol. Flow cytometry was performed on the FACScan (BD Biosciences) and analysis was performed using Cell Quest software (BD Biosciences).

Mice tumor inoculations and measurements. Female athymic nude mice (aged 8–12 weeks) were purchased from Harlan. For tumor inoculations, 100 μL of a 1:1 mixture of Matrigel (BD Biosciences) and RPMI 1640 (GIBCO®) containing 1 x 10^6 MDA-MB-231 cells, expressing either pZeo HA-HuR, pZeo empty vector or wild-type clones, were injected into the left or right mammary fat pad. Tumor volumes were calculated using calipers by measuring using the formula: LxWxDx0.5. Procedures were conducted according to the University of Missouri Columbia Animal Care and Use Committee.

Longitudinal MRI investigation and tumor volume analysis. Magnetic Resonance Imaging (MRI) was performed on a 7T/210 mm Varian Unity Inova MRI system (Varian Inc., Palo Alto, CA) using previously published procedures.[44] Mice were anesthetized with 1–2% isoflurane in oxygen via a nose cone over the entire imaging period. Three mice were imaged weekly for 5 weeks to monitor tumor growth. Mice were imaged to obtain axial planes using multi-slice spin-echo T1-weighted (T1W) imaging sequence applied with a fat-saturation pulse to suppress the strong signals from fatty tissues in the chest. Spin-echo diffusion-weighted imaging (DWI) with b-value = 1,063 s/mm^2 was performed at week 4 to assess the tumor tissue viability, i.e., necrotic tissue or solid tumor tissue. Images were obtained with slice thickness = 0.8 mm and in-plane resolution = 0.059 mm x 0.156 mm. The tumors were manually segmented using VnmrJ software (Varian Inc.,) to obtain the tumor volume in cm^3. DW

images at week 4 were used to differentiate between necrotic tissues and solid tumor tissues.

RNA purification and real-time PCR. RNA was extracted from snap frozen tumors in Trizol reagent (Invitrogen™). One μg of RNA was reverse transcribed and real-time PCR was performed using cDNA (in triplicate) for using SuperScript III two-step qRT-PCR with SYBR green (Invitrogen™). Primers were as follows:

VEGF sense 5'-TTT CTG CTG TCT TGG GTG CAT TGG-3' and antisense 5'-ACC ACT TCG TGA TGA TTC TGC CCT-3', TSP1 sense 5'-TTC CGC CGA TTC CAG ATG ATT CCT-3' and antisense 5'-ACG AGT TCT TTA CCC TGA TGG CGT-3', HIF1α sense 5'-TTG GCA GCA ACG ACA CAG AAA CTG-3' and antisense 5'-TTG AGT GCA GGG TCA GCA CTA CTT-3', GAPDH sense 5'-AGC CTC AAG ATC ATC AGC AAT GCC-3' and antisense 5'-TGT GGT CAT GAG TCC TTC CAC GAT-3'. PCR reactions were performed using Applied Biosystems StepOne PCR system. Results were analyzed using comparative CT method with GAPDH as endogenous reference control.

Microarray. 0.5 μg of total RNA was used to make the biotin-labeled antisense RNA (aRNA) target using the Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX). and hybridized to Illumina HumanWG-6 (V2_0_R3) beadchip (47,000 genes) as per Illumina protocols. After hybridization, the chips were washed and stained with streptavidin-C3 and analyzed by BeadArray reader (Illumina, San Diego, CA).

Histology and imunohistochemistry. Tissue was routinely processed, formalin-fixed and paraffin embedded for hematoxylin-and-eosin-staining and immunohistochemistry. Immunostaining was performed using the avidin-biotin-peroxidase complex method. Slides were incubated at room temperature with the following antibodies: anti-cleaved caspase-3 antibody ([2305-PC-100], Trevigen, Gaithersburg, MD); anti-CD34 (ab8158, MEC 14.7, Abcam, Cambridge, MA); anti-HIF1α (NB100-134, Novus Biologicals, Littleton, CO); anti-VEGF (sc-152 Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and anti-TSP-1 (clone A6.1, MS-420-P1, Thermo Fisher Scientific, Fremont, CA). Slides labeled with anti-CD34 or TSP-1 were incubated with a biotinylated secondary antibody DAKO for CD-34 and rabbit anti-mouse IgG (for TSP-1) followed by streptavidin-linked HRP (DAKO). Cleaved caspase-3 and VEGF slides were incubated with a HRP antibody (EnVision™, DAKO). Bound antibodies were visualized with peroxidase substrates: DAB 3, 3'-diaminobenzidine solution (DAKO) or NovaRED™ (Vector Labs, Burlingame, CA). For the TUNEL apoptosis detection assay we used the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science) and followed the manufacturer's protocol for paraffin sections.

Statistics. All error bars represent standard error of the mean. The p values were calculated using the two-tailed Student *t* test.

Statistical analysis of microarray data. Analysis of microarray gene expression data was primarily performed using the Linear Models for Microarray Data (limma) package[45] and the lumi package,[46] available through the Bioconductor project[47] for use with R statistical software. Quantile normalization was used for between chip normalization. Statistical analysis was performed using moderated t-statistics applied to the log-transformed (base 2) normalized intensity for each gene. Because two measurements were taken from each mouse, the dependency between paired measurements was accounted for by a modified mixed linear model that treated each animal as a block. The within-block correlations were constrained to be equal between genes, and then information was borrowed across genes to moderate the standard deviations between genes via an empirical Bayes method. The contrast of interest computed and tested was the difference between overexpressor and control vector. Adjustment for multiple testing was made using the false discovery rate (FDR) method of Benjamini and Hochberg.[48] We chose 10% as our FDR-cutoff for declaring statistical significance, and a threshold of at least three-fold (up or down) for declaring a biologically significant change in expression. Gene ontology (GO) analyses were carried to test the association between Gene Ontology Consortium terms and the list of differentially expressed genes. In defining the gene universe for the analysis, non-specific filtering was used to increase statistical power without biasing the results. This filtering selected only probes on the Illumina array which had both an Entrez gene identifier,[49] a GO annotation (as provided in the lumiHumanAll.db[50] annotation data package and GO.db[51] annotation maps) and an interquartile range of ≥0.1 on log2 scale across all samples. Using this gene universe, GOstats[52] was used to carry out conditional hypergeometric tests which exploit the hierarchical nature of the relationships among the GO terms for conditioning.[53] We carried out GO analyses for over-representation of biological process (BP), molecular function (MF) and cellular component (CC) ontologies and computed the nominal hypergeometric probability for each GO category. These results were used to assess whether the number of selected genes associated with a given term was larger than expected and a p-value cutoff of 1% was used. GO categories containing less than 10 genes from our gene universe were not considered to be reliable indicators and are not reported.

RNA immunoprecipitation. RNA immunoprecipitation was performed as previously described.[42]

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Note

Supplementry materials can be found at:
www.landesbioscience.com/supplement/GubinCC9-16-sup.pdf

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