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mRNA-BINDING PROTEIN HuR IN BREAST CARCINOGENESIS

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Once we believe in ourselves, we can risk curiosity, wonder, spontaneous delight, or any experience that reveals the human spirit.

poet ee cummings 1894-1962

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ABBREVIATIONS

ADH	atypical ductal hyperplasia
AKT	v-akt murine thymoma viral oncogene homolog 1
AMP	adenosine monophosphate
AMPK	AMP-activted kinase
ARE	AU-rich element
ATF2	activating transcription factor 2
ATM	ataxia telangiectasia mutated
AngII	angiotensin II
ATP	adenosine triphosphate
ATR	ATM and Rad3-related
AUF1	AU-binding factor 1, also known as hnRNP D
Bcl-2	B-cell lymphoma 2
BRCA	breast cancer susceptibility gene
BRF1	butyrate response factor 1
CARM1	coactivator-associated arginine methyltransferase 1
Cdk1	cyclin-dependent kinase 1
Chk1/2	checkpoint kinase 1/2
COX-2	cyclooxygenase 2
CTGF	connective tissue growth factor
DCIS	ductal carcinoma in situ
dCK	deoxy-cytidine kinase
ECM	extracellular matrix
EGF	epidermal growth factor
ELAV	embryonic lethal abnormal vision
EMT	epithelia-to-mesenchymal transition
GM-CSF	granulocyte macrophage colony-stimulating factor
HIF-1α	hypoxia-inducible factor 1α
HNS	HuR nucleocytoplasmic shuttling
HuB	human antigen B, also known as HelN1
HuC	human antigen C
HuD	human antigen D
HuR	human antigen R, also known as HuA
IL	interleukine
IRES	internal ribosomy entry site
JNK	c-Jun N-terminal kinase
KSRP	K homology-splicing regulatory protein
MAPK	mitogen-activated protein kinase
MCC	Merkel cell carcinoma
MCF7	breast cancer cell line
MCT-1	multiple copies in T cell lymphoma 1
MK2	MAPKAPK2, MAPK-activated protein kinase 2
MKP-1	MAPK phosphatase-1
miRNA/miR	microRNA
MMP	matrix metalloproteinase
N.D.	not detected / not determined

NF90	nuclear factor 90
P-bodies	processing bodies
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
pp32/PHAP-1	acidic (leucine-rich) nuclear phosphoprotein 32 family member A
RAB31	Ras oncogene family member
RBP	RNA-binding protein
RCC	renal cell carcinoma
RISC	ribonucleoacid-induced silencing complex
RRM	RNA-recognition motif
SIRT1	sirtuin 1
TDLU	terminal ductal lobular unit
TGF-β	tumor growth factor β
TIA-1	T-cell-restricted intracellular antigen 1
TIAR	TIA1-related protein
TNF-α	tumor necrosis factor α
TNM	tumor-node-metastasis
TSP1	tumor suppressor and angiogenesis inhibitor 1
TTP	tristetraproline
TTR-RBP	turnover and/or translation regulatory RNA-binding protein
uPA	urokinase
uPAR	urokinase receptor
UTR	untranslated region
UV	ultraviolet
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications. Articles are referred to in the text by their Roman numerals:

I **Heinonen M**, Bono P, Narko K, Chang S-H, Lundin J, Joensuu H, Furneaux H, Hla T, Haglund C, and Ristimäki A. Cytoplasmic HuR expression is a prognostic factor in invasive ductal breast carcinoma. Cancer Research, 65(6): 2157-2161 (2005).

II **Heinonen M**, Fagerholm R, Aaltonen K, Kilpivaara O, Aittomäki, K, Blomqvist C, Heikkilä P, Haglund C, Nevanlinna H, and Ristimäki A. Prognostic role of HuR in hereditary breast cancer. Clinical Cancer Research, 13(23): 6959-6963 (2007).

III **Heinonen M**, Hemmes A, Salmenkivi K, Abdelmohsen K, Vilén ST, Laakso M, Leidenius M, Salo T, Hautaniemi S, Gorospe M, Heikkilä P, Haglund C, and Ristimäki A. Role of RNA binding protein HuR in ductal carcinoma *in situ* of the breast. *In Press* The Journal of Pathology (2011).

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ABSTRACT

Breast cancer is the most common cancer among women. Although its prognosis has improved nowadays, methods to predict the progression of the disease or to treat it are not comprehensive. This thesis work was initiated to elucidate in breast carcinogenesis the role of HuR, a ubiquitously expressed mRNA-binding protein that regulates gene expression posttranscriptionally. HuR is predominantly nuclear, but it shuttles between the nucleus and the cytoplasm, and this nucleocytoplasmic translocation is important for its function as a RNA-stabilizing and translational regulator. HuR has been associated with diverse cellular processes, for example carcinogenesis. The specific aims of my thesis work were to study the prognostic value of HuR in breast cancer and to clarify the mechanisms by which HuR contributes to breast carcinogenesis. My ultimate goal is, by better understanding the role of HuR in breast carcinogenesis, to aid in the discovery of novel targets for cancer therapies.

HuR expression and localization was studied in paraffin-embedded preinvasive (atypical ductal hyperplasia, ADH, and ductal carcinoma *in situ*, DCIS) specimens as well in sporadic and familial breast cancer specimens. Our results show that cytoplasmic HuR expression was already elevated in ADH and remained elevated in DCIS as well as in cancer specimens. Clinicopathological analysis showed that cytoplasmic HuR expression associated with the more aggressive form of the disease in DCIS, and in cancer specimens it proved an independent marker for poor prognosis. Importantly, cytoplasmic HuR expression was significantly associated with poor outcome in the subgroups of small (< 2 cm) and axillary lymph node-negative breast cancers. HuR proved to be the first mRNA stability protein the expression of which is associated in breast cancer with poor outcome.

To explore the mechanisms of HuR in breast carcinogenesis, lentiviral constructs were developed to inhibit and to overexpress the HuR expression in a breast epithelial cell line (184B5Me). Our results suggest that HuR mediates breast carcinogenesis by participating in processes important in cell transformation, in programmed cell death, and in cell invasion. Global gene expression analysis shows that HuR regulates genes participating in diverse cellular processes, and affects several pathways important in cancer development. In addition, we identified two novel target transcripts (connective tissue growth factor, CTGF, and Ras oncogene family member 31, RAB31) for HuR.

In conclusion, because cytoplasmic HuR expression in breast cancer can predict the outcome of the disease it could serve in clinics as a prognostic marker. HuR accumulates in the cytoplasm even at its non-invasive stage (ADH and DCIS) of the carcinogenic process and supports functions essential in cell alteration. These data suggest that HuR contributes to carcinogenesis of the breast epithelium.

INTRODUCTION TO THE SUBJECT

1. FEMALE MAMMARY GLAND

The female mammary gland is a unique organ since it fully develops only after puberty and undergoes constant remodeling and differentiation during the reproductive phase. Before puberty the development of mammary glands does not differ between females and males. However, at puberty the female mammary gland starts differentiating under the influence of hormones, predominantly estrogen derived from the ovaries. The ducts start to elongate and branch, forming a ductal tree with 15 to 25 major ducts (Fridriksdottir *et al.* 2005), each having an opening to the nipple. These main ducts branch further, eventually leading to terminal ductal lobular units (TDLUs) which terminates to individual blunt-ending ductuoles (acini) forming a lobule (Figure 1) (Howard *et al.* 2000, Parmar *et al.* 2004).

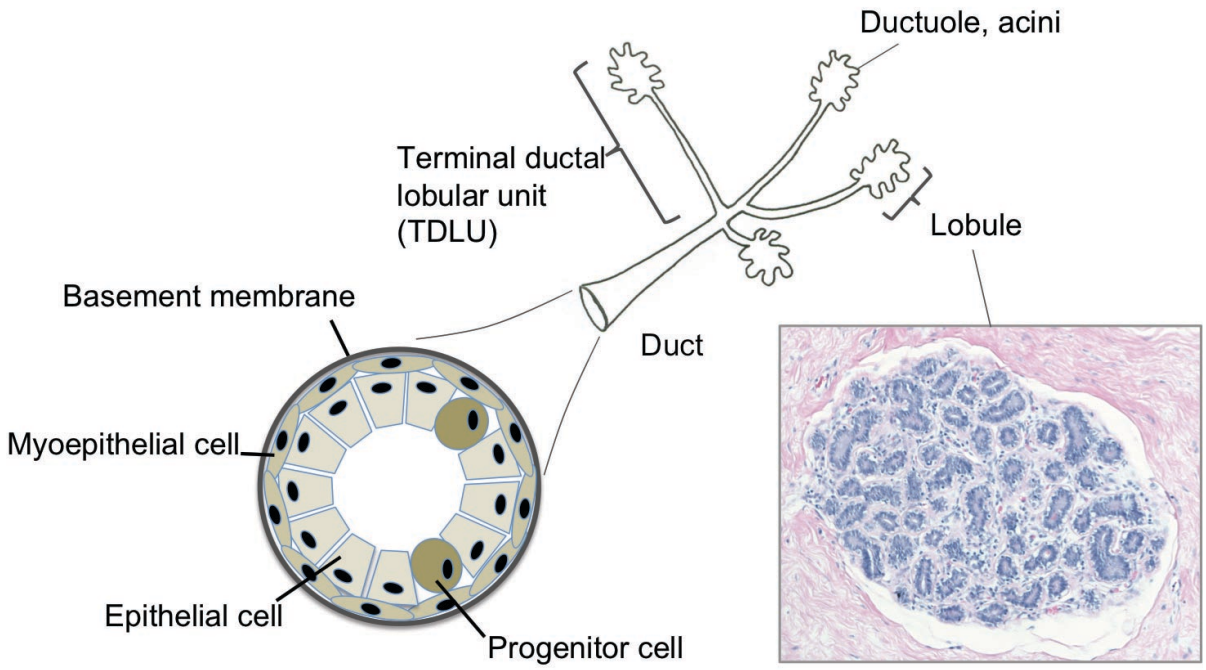


Figure 1. Schematic of a cross-section of a duct and part of a ductal tree ending in a TDLU. Immunohistochemical breast tissue stained with Mayer's hematoxylin and eosin. Original magnification is 100x.

Acini in TDLUs are responsible for milk secretion during lactation (Gudjonsson *et al.* 2005). Ducts and lobules form from two layers of epithelial cells, an outer layer of basal myoepithelial cells and an inner layer of polarized luminal cells separated from the surrounding stroma (connective and fat tissue) by a basement membrane (Figure 1) (Gudjonsson *et al.* 2005). Additionally, the luminal epithelial side contains breast epithelial

progenitor cells, precursors for both types of epithelial cells. These progenitor cells are responsible for the continuous renewal, growth, and branching in the breast as a response to hormonal and growth factor signals during the normal menstrual cycle, in pregnancy, and at postmenopausal involution (Howard *et al.* 2000, Gudjonsson *et al.* 2005).

2. BREAST CARCINOGENESIS

Breast cancer is the most common cancer among women. In 2009 in Finland 4464 new breast cancer cases occurred in women, comprising 32% of all new cancer cases in women (www.cancer.fi/syoparekisteri). The worldwide estimate in 2008 was for 12.7 million new cancer cases and 7.6 million cancer-related deaths. The most commonly diagnosed cancer was pulmonary (12.7%), but breast cancer (10.9%) was in second place (http://globocan.iarc.fr), being by far the most frequent cancer among women, with an estimated 1.38 million new cases diagnosed in 2008 (23% of all cancers in women). Australia, Europe, and North America are the high-risk areas, where 6% of women are estimated to develop invasive breast cancer before the age of 75 (Tavassoli *et al.* 2003). However, the prognosis has improved, and 89% of the patients in Finland are still alive 5 years after diagnosis (www.cancer.org, www.cancer.fi). The exact etiology of breast cancer is unknown. However, reasons for breast cancer development are multifactorial, involving reproductive factors and hormones, as well as lifestyle and diet (Tavassoli *et al.* 2003). Known predisposing and protective factors against breast cancer are summarized in Table 1.

Table 1. Predisposing and protective factors against breast cancer.

Female breast cancer	
Predisposing factors	**Protective factors**
Family history of breast cancer Benign breast disease Exposure to ionizing radiation Early menarche Late menopause Childlessness Oral contraceptives Abundant alcohol consumption Postmenopausal obesity Rapid growth and greater adult height Low physical activity	Early age at full-term pregnancy Breast-feeding Vitamin D High intake of fresh fruits and vegetables High intake of fiber

Transforming genetic and epigenetic events in a single cell initiate breast carcinogenesis. Progression of the disease results from additional accumulation of mutations combined with clonal growth and selection of the cells, leading to heterogeneous disease with a broad variety of pathological appearance and diverse clinical outcomes (Simpson *et al.* 2005). To be able to

treat patients appropriately, ways to identify the disease are necessary. The World Health Organization classifies breast epithelial tumors into 19 subgroups based on their histological appearance. Factors both prognostic and predictive, like tumor-node metastasis (TNM) stage, and expression of hormonal and growth factor receptors provide information on how to treat patients and predict disease progression. Recently, gene expression arrays have been introduced as a model to study breast cancer biology, improve diagnosis, identify prognostic signatures, predict cancer response to adjuvant therapy, and identify therapeutic targets (Cheang *et al.* 2008). These prognostic and predictive factors or gene expression profiling methods are, however, not comprehensive, and new markers and methods to predict disease development are vital.

REVIEW OF THE LITERATURE

1. POSTTRANSCRIPTIONAL GENE REGULATION

Regulation of eukaryotic gene expression is a tightly controlled process that can occur at multiple levels consisting of transcription, RNA processing, mRNA transport, turnover, storage, and translation. Transcription is the best-known regulatory method of eukaryotic gene expression, but the role of posttranscriptional regulation is emerging. Two main classes of RNA-binding factors, RNA-binding proteins (RBPs) and small noncoding RNAs, i.e. microRNAs (miRNAs), and short interfering RNAs, control the posttranscriptional gene regulation.

In vertebrates, the stability of mRNA can range from less than an hour to over 12 hours (Sharova *et al*. 2009), leading to a great difference in the abundance of transcripts. The half-lives of mRNAs are dependent on *cis*-acting elements in the mRNA and *trans*-acting factors recognizing these elements. The association of RBPs and miRNAs determines the fate of the mRNA and their recruitment to different sites in the cell, like processing bodies (P-bodies), stress-granules, polysomes, exosomes, and RNA-induced silencing complex (RISC), each specialized in distinct aspects of mRNA metabolism (Figure 2). One such *cis*-acting element in the mRNA is a turnover and translation regulatory element that *trans*-acting RBPs recognize.

1.1 Turnover and translation regulatory RNA-binding proteins (TTR-RBPs)

TTR-RBPs recognize and bind to specific *cis*-acting elements in mRNA sequences frequently located in the 5' and the 3' untranslated regions (UTR), causing alterations in mRNA stability or translation efficiency or both (Pullmann *et al*. 2007). Conventionally, these elements in the mRNAs have been called AU-rich elements (AREs), since the first stability elements found were enriched with adenylate and uridylate (AU) or uridylate (U) stretches (Brennan *et al*. 2001). TTR-RBPs are a heterogeneous group of proteins with tightly connected functions, and their influence on the same target mRNA may be competitive or cooperative (Table 2). Tristetraproline (TTP), AU-binding factor 1 (AUF1, also called hnRNP D), the K homology splicing regulatory protein (KSRP), nuclear factor 90 (NF90), human antigen R (HuR), T-cell-restricted intracellular antigen 1 (TIA-1), and TIA-1-related protein (TIAR) can all associate with its cognate mRNA and with other TTR-RBPs mRNAs and regulate their expression (Tchen *et al*. 2004, Pullmann *et al*. 2007). TTP, AUF1, KSRP, and butyrate response factor 1 (BRF1) have all been primarily associated with mRNA decay. Human antigen (Hu) proteins (HuR, HuB, HuC, HuD) and NF90 can promote mRNA stability or affect translation of their target transcripts or both. TIA-1 and TIAR have been shown to inhibit translation. Additionally, nucleolin, αCP1, RNPC1, CUG-BP2, and PAIP2 have also

been suggested to promote stability of their target transcripts and CUG-BP1 to enhance translation. Their functions are, however, not well characterized (Abdelmohsen *et al*. 2008a).

Table 2. TTR-RBPs regulate the same target genes, and their effect on mRNA may be competitive or cooperative.

TTR-RBP	Target mRNAs*	Effect on mRNA
TTP	GM-CSF, c-fos, TNF-α, COX-2, IL-2, VEGF, IL-8, interferon-γ, TTP	mRNA decay
AUF1	Cyclin D1, c-fos, GM-CSF, TNF-α, IL-1β, IL-3, p16, p21, COX-2	mRNA decay
	c-Myc	Increase in translation
KSRP	c-fos, c-jun, iNOS, TNF-α, IL-2	mRNA decay
BRF1	TNF-α, IL-3	mRNA decay
NF90	MKP-1, VEGF, IL-2, p21, MyoD	mRNA stabilization
TIA-1	COX-2, TNF-α, CASP8, cytochrome c	Decrease in translation
TIAR	COX-2, c-myc, EIF4A, EIF4E, MMP-13	Decrease in translation
HuR	c-fos, p21, cyclins A2/B1/E1/D1, iNOS, GM-CSF, VEGF, SIRT1, TNF-α, Bcl-2, Mcl-1, COX-2, uPA, uPAR, IL-3, MKP-1, p53, HIF-1α, Mdm-2, Snail, MMP-9, eIF4E	mRNA stabilization
	p53, ProTα, cytochrome c, MKP-1, cyclin A2, Bcl-2, VEGF, TSP-1	Increase in translation
	p27, IGF-IR, Wnt5a, TNF-α, c-Myc	Decrease in translation

* References: TTP (Sully *et al*. 2004, Tchen *et al*. 2004, Ogilvie *et al*. 2005, 2009, Hau *et al*. 2007, Suswam *et al*. 2008), AUF1 (Cok *et al*. 2004, Lal *et al*. 2004, Wang *et al*. 2005, Liao *et al*. 2007, Abdelmohsen *et al*. 2008a, Chang *et al*. 2010), KSRP (Linker *et al*. 2005, Abdelmohsen *et al*. 2008a), BRF1 (Stoecklin *et al*. 2002, Abdelmohsen *et al*. 2008a), NF90 (Shim *et al*. 2002, Shi *et al*. 2005, Abdelmohsen *et al*. 2008a, Kuwano *et al*. 2008b), TIA-1 (Piecyk *et al*. 2000, Lopez de Silanes *et al*. 2005, Kawai *et al*. 2006), TIAR (Yu *et al*. 2003, Mazan-Mamczarz *et al*. 2006, Liao *et al*. 2007, Abdelmohsen *et al*. 2008a), HuR (Levy *et al*. 1998, Wang *et al*. 2000a, 2000b, Dean *et al*. 2001, Ming *et al*. 2001, Kullmann *et al*. 2002, Akool *et al*. 2003, Mazan-Mamczarz *et al*. 2003, 2008a, Sengupta *et al*. 2003, Tran *et al*. 2003, Lal *et al*. 2004, 2005, Winzen *et al*. 2004, Linker *et al*. 2005, Meng *et al*. 2005, Guo *et al*. 2006, Kawai *et al*. 2006, Leandersson *et al*. 2006, Abdelmohsen *et al*. 2007a, 2007b, Dong *et al*. 2007, Galban *et al*. 2008, Kuwano *et al*. 2008b, Ghosh *et al*. 2009, Kim *et al*. 2009, Topisirovic *et al*. 2009, Doller *et al*. 2010, Kakuguchi *et al*. 2010, Kurosu *et al*. 2011).

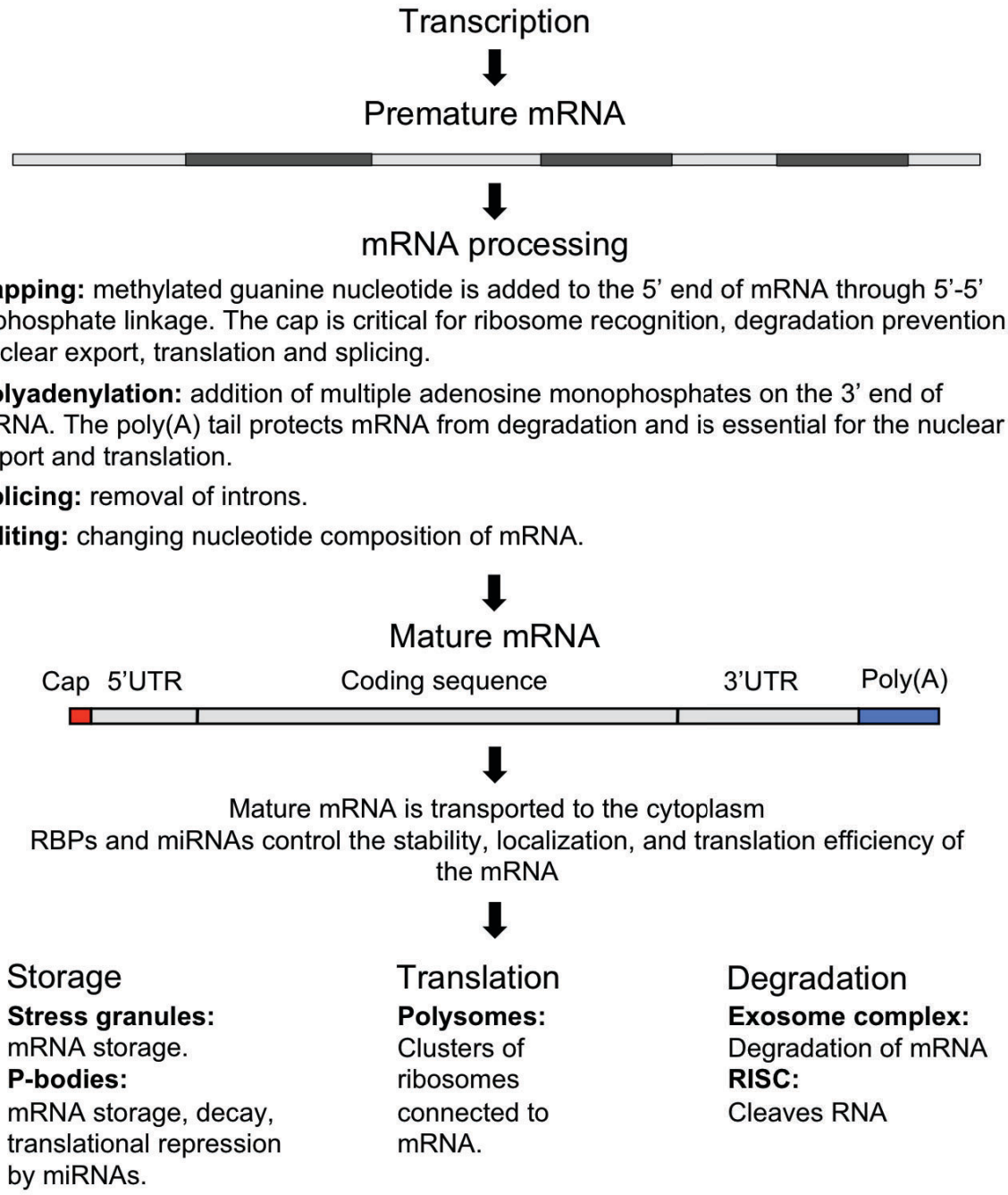
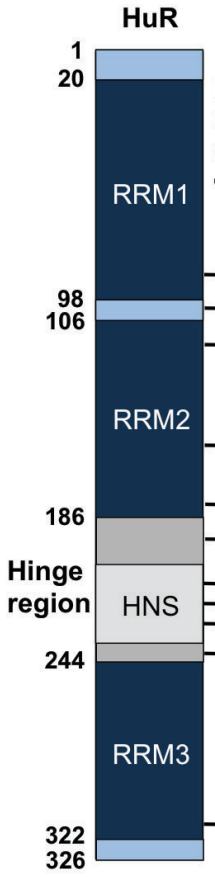


Figure 2. Posttranscriptional regulation of RNA. After transcription, premature mRNA is processed to mature mRNA, and transported to the cytoplasm, where RBPs and miRNAs participate in the regulation of mRNA translation, degradation, and storage.

2. HUMAN ANTIGEN R, HUR

HuR (HuA) is a member of the embryonic lethal abnormal vision (ELAV)-like / Hu-protein family of RBPs. This protein family consists of ubiquitously expressed HuR protein and three other family members, HuB/HelN1, HuC, and HuD, that are mainly expressed in the neuronal tissues. The *Elav* gene was first discovered in *Drosophila*, where it regulates the development and maintenance of the nervous system. Human HuR was first cloned and characterized by Ma *et al.* in 1996. HuR is localized to human chromosome 19p13.2, and the protein consists of 326 amino acids having a molecular mass of 36 kD. HuR shares over 90% amino acid similarity with its other family members (Ma *et al*. 1996, 1997).

HuR consists of three RNA recognition motifs (RRM) and a hinge region where the HuR nucleocytoplasmic shuttling (HNS) sequence is located (Figure 3). Through RRM1 and RRM2, HuR binds to and mediates recognition of its target transcripts. RRM3 is thought to bind the poly(A) tail and maintain stability of the RNA-protein complex (Brennan *et al*. 2001, Hinman *et al*. 2008). Like other TTR-RBP, HuR recognizes elements rich in AU or U (AREs) usually located in the 5' and/or 3'-UTR of its target transcripts, mediating their stabilization or translation or both (Brennan *et al*. 2001, Lopez de Silanes *et al*. 2004). The exact mechanisms by which HuR stabilizes or regulates translation of target mRNAs are not completely understood. However, HuR probably competes with other RBPs, and the net effect of the RBPs then resolve if the mRNA is stabilized or degraded (Abdelmohsen *et al*. 2010a). The translational control of HuR has been proposed to cause its effect through internal ribosomal entry sites (IRES) in the 5'UTRs of its targets (Kullmann *et al*. 2002), or to compete or cooperate with miRNAs (Bhattacharyya *et al*. 2006, Kim *et al*. 2009). However, stability- and translation-independent roles of HuR have also been suggested, i.e. functioning as a splicing factor and apoptosis inducer (Izquierdo 2008, Mazroui *et al*. 2008, von Roretz *et al*. 2010). Through its target transcripts, HuR participates in diverse biological processes in the cell like transformation, immune response, cell survival, inflammation, cell cycle control, differentiation, and senescence (Abdelmohsen *et al*. 2007a, 2008a, 2010a, Masuda *et al*. 2009). The chromosomal locus of HuR is associated with a number of translocations and oncogenic gains in human tumors (Ma *et al*. 1997). However, primarily alterations in HuR function depend neither on protein abundance, nor on mutations of the gene, nor on alterations in its copy number, but on subcellular localization of HuR and the binding of HuR to its target transcripts.



Position of modified amino acid	Type of post-translational modification	Enzyme performing the modification	Influence on HuR function	Reference
S88	Phosphorylation	Chk2	RNA binding	1
S100	Phosphorylation	Chk2	RNA binding	1
T118	Phosphorylation	Chk2, $p38^{MAPK}$	RNA binding, localization	1, 2
S158	Phosphorylation	PKCα	RNA binding, localization	3
K182	Ubiquitinylation	n.d.	Protein stability	4
S202	Phosphorylation	Cdk1	Localization	5
R217	Methylation	CARM1	RNA binding, localization	6
S221	Phosphorylation	PKCα, PKCδ	RNA binding, localization	3, 7
D226	Cleavage	Caspases	Protein integrity	8
S242	Phosphorylation	n.d.	Localization	9
S318	Phosphorylation	PKCδ	RNA binding, localization	7

Figure 3. Schematic of HuR protein with posttranslational modification information. HuR consists of three RNA recognition motifs (RRM) and a hinge region (amino acids 186-224) containing the HuR nucleocytoplasmic shuttling sequence (HNS). Modified from the article by Abdelmohsen *et al*. 2010a. References: 1. Abdelmohsen *et al*. 2007, 2. Lafarga *et al*. 2009, 3. Doller *et al*. 2007, 4. Abdelmohsen *et al*. 2009, 5. Kim *et al*. 2008, 6. Li *et al*. 2002, 7. Doller *et al*. 2008, 8. Mazroui *et al*. 2008, 9. Kim *et al*. 2008.

2.1 Nucleocytoplasmic shuttling of HuR

Although HuR is predominantly nuclear, its role in the nucleus has remained rather indistinct. HuR is associated with mRNA splicing and plays a role in polyadenylation and export of its target transcripts from the nucleus (Izquierdo 2008, Papadopoulou *et al*. 2010). However, the cytoplasmic localization of HuR has been associated with its ability to stabilize or mediate translation of its target transcripts, and thus in the cell it participates in diverse biological processes. Nucleocytoplasmic translocation of HuR can occur by at least two independent mechanisms and is influenced by kinases: cyclin-dependent kinase 1 (Cdk1, G2-phase kinase also known as cell division cycle 2), AMP-activated protein kinase (AMPK), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) p38 that again react with various endogenous and external stimuli such as hormones, cytokines, growth factors, UV radiation, hypoxia, lack of nutrients, and oxidative stress (Doller *et al*. 2008b).

HNS is located between RRM2 and -3, and contains a bidirectional shuttling signal that enables nuclear export and importation of HuR (Figure 3). Transport receptors transportin-1 and -2 and importin-1α recognize the HNS and mediate the translocation of HuR across the nuclear membrane through nuclear pore complexes (Gallouzi *et al*. 2001b, Rebane *et al*. 2004, Wang *et al*. 2004). Adapter proteins pp32 and acidic protein rich in leucine also bind HuR and mediate its transportation via the nuclear export receptor chromosome maintenance region 1 (Gallouzi *et al*. 2001a). What determines the HuR shuttling pathway is unknown, but most likely HuR, its target transcript, and an activating signal all play a role.

2.2 Signaling pathways related to HuR regulation

Cytoplasmic shuttling of HuR is modulated by cellular signaling cascades including MAPK and its downstream kinase MK2, AMPK, and the PKC family. Additionally, Cdk1 has been connected with UVC-triggered HuR shuttling (Doller *et al*. 2008b).

AMPK is an essential regulator of cell metabolism. It is activated by the increase in the AMP/ATP-ratio caused for example by lack of nutrients or by physical exercise. Activation of AMPK leads to the inhibition of energy-conserving pathways and the activation of catabolic pathways that produce ATP. AMPK does not phosphorylate HuR directly, but activated AMPK phosphorylates (S105) and acetylates (K22) importin-α1, a nuclear transportation protein, leading to the nuclear accumulation of HuR (Figure 4) (Wang *et al*. 2004).

Ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR) kinases detect DNA damage. ATM is mainly activated by double-strand breaks in DNA caused by IR, whereas ATR can be activated by damaging agents such as UV and chemical inhibitors of DNA replication (Yang *et al*. 2004, Kim *et al*. 2010). ATM/ATR phosphorylates checkpoint kinase (Chk) 1, which further leads to phosphorylation of Cdk1 and inactivation of this kinase (Kim *et al*. 2010). Silencing of Cdk1 increases cytoplasmic levels of HuR and its interaction with its target transcripts. For contrast, Cdk1 can phosphorylate HuR at position serine (S) 202 during the G2/M phase and promote the nuclear localization of HuR (Figure 4) (Kim *et al*. 2008a).

MAPKs are serine/threonine-specific protein kinases activated by several extracellular signals, thus leading to various cellular activities such as gene expression, mitosis, differentiation, proliferation, and cell survival and apoptosis (Figure 4) (Keshet *et al*. 2010). MAPKs increase cytoplasmic localization of HuR and so participate in stabilization or translation or both of tumor necrosis factor α (TNF-α), interleukine (IL) 6 and IL-8, cyclooxygenase 2 (COX-2), c-fos, granulocyte macrophage colony-stimulating factor (GM-CSF), urokinase (uPA), and urokinase receptor (uPAR) (Doller *et al*. 2008b). Interestingly, HuR also stabilizes and facilitates translation of MAPK phosphatase-1, MKP-1 (Kuwano *et al*. 2008a), a protein that dephosphorylates and inactivates MAPKs extracellular-signal regulated kinase, c-Jun N-terminal kinase (JNK), and p38. A negative feedback loop has been

suggested in which a stress signal activates MAPKs, leading to activation of transcription factors that increase activity of stress-response genes. In this model, MAPK-activated HuR stabilizes and enhances translation of stress-response mRNAs, including MKP-1, which production eventually leads to shut-off of MAPKs.

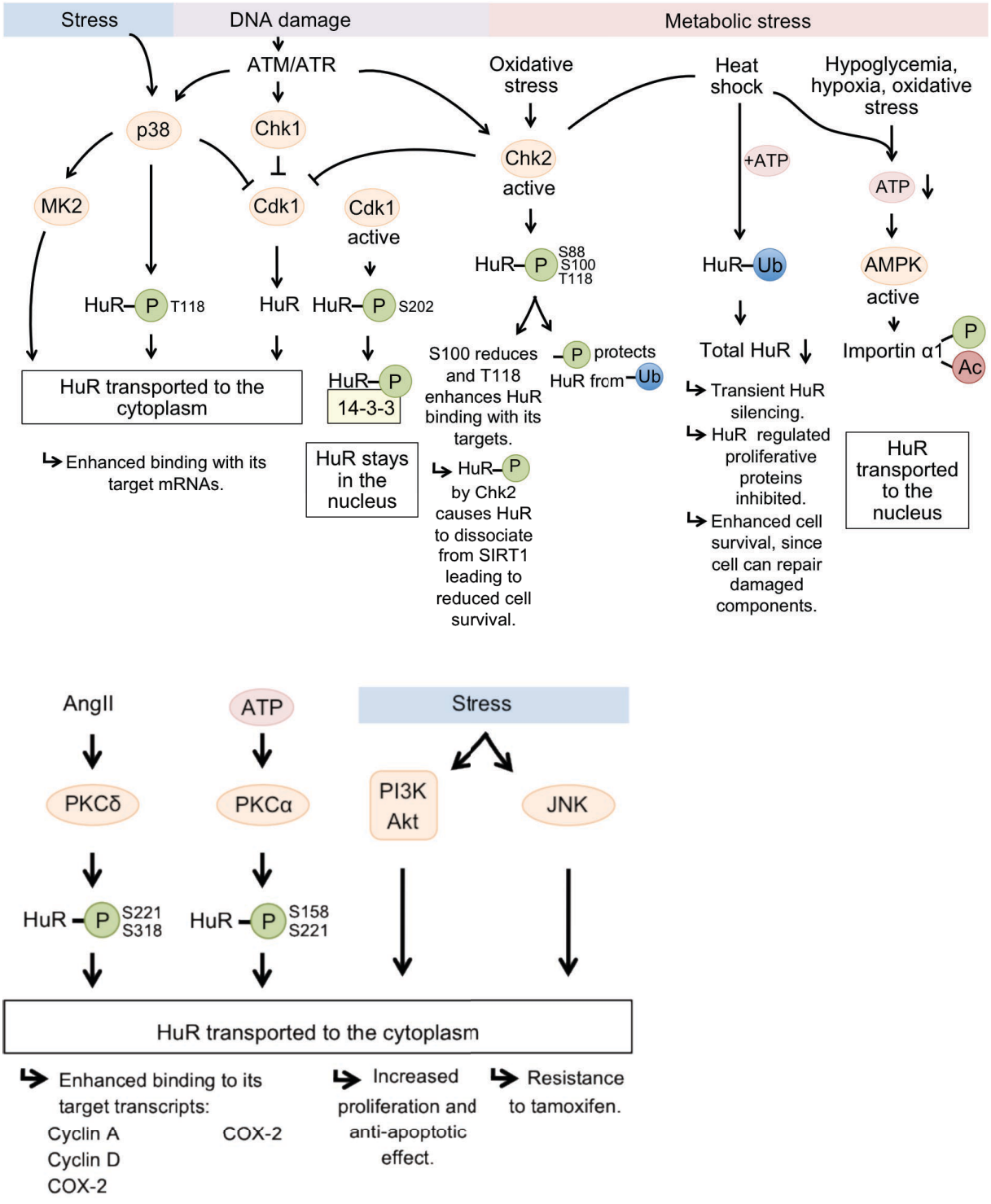


Figure 4. Signaling pathways related to HuR regulation.

PKCα and PKCδ are members of a PKC family of serine/threonine protein kinases consisting of at least 10 different isoforms (Doller *et al*. 2008b). PKCs are related to several biological processes including development, differentiation, proliferation, and carcinogenesis, and respond to a variety of signals including hormones and peptides such as Angiotensin II (AngII), epidermal growth factor (EGF), and ATP (Doller *et al*. 2007, 2008a). PKCα can phosphorylate HuR at the sites of S158 and S221, and PKCδ at the sites of S221 and S318 (Doller *et al*. 2007, 2008a, 2010). PKCα- or PKCδ-mediated serine-phosphorylation of HuR leads to nucleocytoplasmic shuttling of HuR, and to increased stabilization of its target transcripts (e. g. COX-2, cyclin D1 and cyclin A; Figure 4).

Additionally, a still-unidentified kinase can phosphorylate HuR at the site of S242, which prevents its cytoplasmic localization and posttranscriptional regulation of cyclin A2 and B1 (Kim *et al*. 2008b).

2.3 MicroRNAs regulating HuR expression

MicroRNAs (miRNAs/miR) are short ~22 nucleotide long non-coding RNA molecules that participate in mRNA regulation by activating an endonuclease cleavage, inhibiting translation or enhancing mRNA decapping. MiRNAs regulate several biological processes such as development, cell differentiation, the cell division cycle, and apoptosis, as well as cancer (Garzon *et al*. 2009). They are generated from a stem-loop structure forming precursor transcripts that are transported to the cytoplasm and cleaved by Dicer, an endoribonuclease in the RNase III family. Matured miRNAs accumulate in the RISC and function primarily as repressors of mRNA stability or translation or both (Valencia-Sanchez *et al*. 2006). Thus far, four miRNAs have been shown to repress HuR expression in cells (Table 3). MiR-16, miR-125a, and miR-519 inhibit translation of HuR, while miR-34 inhibits both stability and translation of HuR expression (Abdelmohsen *et al*. 2008b, 2010b, Guo *et al*. 2009, Kojima *et al*. 2010, Xu *et al*. 2010). MiR-519 represses HuR translation without affecting HuR mRNA abundance, resulting in reduced proliferation of the cells (Abdelmohsen *et al*. 2008b). In comparison to cancers and adjacent tissues of lung, ovary, and kidney specimens, cancers show markedly higher levels of HuR expression and lower miR-519 levels than do their healthy tissue controls (Abdelmohsen *et al*. 2010b). MiR-16 and -125a inhibit HuR translation in breast cancer cells, and similar to miR-519, miR-16 is inhibited in breast cancer specimens (Xu *et al*. 2010). Re-establishment of miR-125a in breast cancer cells inhibits cell growth by restraining proliferation and inducing apoptosis (Guo *et al*. 2009). MiR-34a inhibition of HuR expression leads to repression of its downstream targets sirtuin 1 (SIRT1) and B-cell lymphoma 2 (Bcl-2) in prostate cancer cells. Inhibition of miR-34a expression, in contrast, leads to paclitaxel resistance via up-regulation of SIRT1 and Bcl-2 in the prostate cells (Kojima *et al*. 2010). These results thus show that miRNAs are important regulators of HuR expression and may prove to be functional in cancer treatments as tumor repressors.

Table 3. MicroRNAs in HuR regulation.

miRNA	**miRNA expression in cancers**	**miRNA effect on HuR**	**Experimental data ***
miR-16	Inhibited in breast tumors	Inhibit translation	miR-16 inhibits HuR expression leading to inhibition of COX-2, c-fos, and SIRT1.
miR-34a	Inhibited in several cancers, e.g. breast	Inhibit mRNA stability and translation	miR-34a inhibits HuR expression leading to inhibition of Bcl-2 and SIRT1.
miR-125a	Inhibited in breast cancer	Inhibit translation	miR-125a inhibits HuR expression and suppresses cell growth by inducing apoptosis and reducing cell migration.
miR-519	Reduced expression in lung, kidney and ovarian cancers	Inhibit translation	miR-519 alters cell proliferation by regulating HuR protein abundance.

* References: Iorio *et al*. 2005, Abdelmohsen *et al*. 2008b, 2010b, Guo *et al*. 2009, Kojima *et al*. 2010, Xu *et al*. 2010, Vogt *et al*. 2011.

2.4 Binding of HuR to its target transcripts

HuR undergoes modifications that alter its binding to its target transcripts (Figure 3). HuR can be phosphorylated (Abdelmohsen *et al*. 2007b, Doller *et al*. 2007, 2008a), methylated (Li *et al*. 2002), cleaved (Mazroui *et al*. 2008) and ubiquitinylated (Abdelmohsen *et al*. 2009). Additionally, HuR mRNA contains multiple polyadenylation sites (Al-Ahmadi *et al*. 2009).

Eight recently discovered phosphorylation sites in the HuR protein sequence affect the binding affinity of HuR to its target transcripts or subcellular localization of HuR or both (Figures 3 and 4). HuR can be phosphorylated at the sites of S88, S100, S158, S202, S221, S242, S318, and threonine (T) 118. Kinases mediating these phosphorylations are Chk2, p38 MAPK, PKC, and Cdk1 (Abdelmohsen *et al*. 2007b, Doller *et al*. 2007, 2008a, Lafarga *et al*. 2009). In oxidative stress, Chk2 phosphorylates HuR on sites S88, S100, and T118, causing HuR to dissociate from its target mRNA SIRT1, which leads to degradation of SIRT1 mRNA, lowering of the protein abundance, and finally to reduced in cell survival (Abdelmohsen *et al*. 2007b). Similarly, phosphorylation of HuR at the site of T118 by p38 MAPK improves binding of HuR to $p21^{Cip1}$, leading to an effective G1 arrest of the cells (Lafarga *et al*. 2009). Phosphorylation of HuR by PKCα at the sites of S158 and S221 enhances HuR binding to COX-2 mRNA, and PKCδ phosphorylation at the sites of S221 and S318 causes enhanced binding of HuR to COX-2, cyclin A, and D1 mRNAs in the cells (Doller *et al*. 2007, 2010).

Methylation of HuR at the site of R217 and to a lesser extent at the site of R206 (both located in the hinge region) is mediated by coactivator-associated arginine methyltransferase 1, CARM1 (Figure 3) (Li *et al*. 2002). The exact meaning of HuR methylation is still unclear, but results from differentiating rat liver cells suggest that HuR methylation causes target transcript destabilization or inhibition of its translation or both (Vazquez-Chantada *et al*. 2010). Ubiquitination of HuR at the site of K182 reduces HuR protein abundance, leading to a decrease in HuR target mRNAs related to cell proliferation, and to enhancement of cell survival by allowing cells to repair heat-damaged components (Abdelmohsen *et al*. 2009). As a response to lethal stress, HuR is transported to the cytoplasm with pp32/PHAP-I, after which caspase-3 and -7 cleavage HuR at the site of D226, producing fragments of 24 kD and 9 kD. This cleavage of HuR promotes cell death (Mazroui *et al*. 2008).

HuR mRNA 3'UTR consists of multiple polyadenylation sites (1.5-, 2.7-, 6-kb) that produce alternative variants with differing stability elements. The long 6-kb HuR mRNA contains a typical class I ARE sequence (AUUUA) and is more labile than the 1.5- and 2.7-kb 3'UTR isoforms lacking the classical ARE and having ATTAAA and U-rich stretches in their sequences (Al-Ahmadi *et al*. 2009). HuR binds to and regulates its own mRNA, but other TTR-RBPs like AUF1, TTP, NF90, TIA-1, and TIAR also bind HuR. These variants respond differently to HuR-mediated stabilization, when differences in the stability elements cause divergence in the abundance of HuR transcripts in the cells (Al-Ahmadi *et al*. 2009). This can have an effect on tissue-specific expression, mRNA localization, mRNA decay, and translation.

3. HUR IN CARCINOGENESIS

HuR has been associated with diverse roles in the cells including differentiation, cell response to damaging stimuli, and immune and inflammatory responses, as well as carcinogenesis. HuR can mediate functions considered hallmarks of cancer (Hanahan *et al*. 2011), i.e. promote the expression of proteins that increase proliferation, enhance cell survival, reduce apoptosis, improve angiogenesis, reduce immune recognition, and facilitate invasion and metastasis (Figure 5).

3.1 HuR regulates mRNAs encoding proteins involved in cancer development

A cancer cell must gain the ability to promote proliferation in order to grow into a tumor mass. HuR enhances the stability and raises the levels of many cyclins, which activate cyclin-dependent kinases at different cell cycle phases, and cause cell division to shorten and tumor mass to increase. HuR promotes the expression of cyclin D1, cyclin E1, cyclin A2, cyclin B1, EGF and eukaryotic translation initiation factor (Wang *et al*. 2000a, Lal *et al*. 2004, Sheflin *et al*. 2004, Guo *et al*. 2006, Topisirovic *et al*. 2009, Kakuguchi *et al*. 2010). Additionally, HuR can inhibit translation of p27, a factor that controls Cdk2 and prevents cell cycle transition to

the S phase (Kullmann *et al*. 2002).

To be able to survive, tumor cells must defeat death-causing signals. HuR promotes expression of many anti-apoptotic proteins (Prothymosin-α (Pro-Tα), Bcl-2, myeloid cell leukemia sequence 1 (Mcl-1), SIRT1, p21, Mdm2) and represses the production of a pro-apoptotic protein (c-Myc), thus having an anti-apoptotic effect on cancer cells (Wang *et al*. 2000b, Lal *et al*. 2005, Abdelmohsen *et al*. 2007a, 2007b, Ghosh *et al*. 2009, Kim *et al*. 2009). Furthermore, HuR promotes exon 6 skipping on Fas mRNA by binding to an exon-splicing silencer. Exclusion of exon 6 by an alternative RNA splicing of the primary transcript of the apoptosis receptor Fas produces a soluble isoform that prevents apoptosis (Izquierdo 2008, 2010). What is important to notice, however, is that under prolonged and lethal cell stress, HuR is translocated to the cytoplasm together with apoptosome activator pp32/PHAP-I, and goes through cleavage that accelerates the progress of apoptosis (Mazroui *et al*. 2008). Although HuR facilitates translation of p53 (Mazan-Mamczarz *et al*. 2003), a powerful pro-apoptotic protein, HuR has a strong anti-apoptotic effect on cancer cells via its other targets, leading to greater survival of cancer cells.

When a tumor grows, cancer cells need to extend the local vasculature to protect delivery of nutrients and oxygen. HuR has been shown to affect the expression of pro-angiogenic factors like hypoxia-inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF), and COX-2, leading to an increase in tumor vasculature (Levy *et al*. 1998, Sheflin *et al*. 2004, Galban *et al*. 2008, Kurosu *et al*. 2011). HuR enhances the expression of HIF-1α and VEGF that are strongly expressed at low oxygen concentrations, and increases the stability and translation of COX-2 mRNA (Dixon *et al*. 2001, Nabors *et al*. 2001), a key enzyme in the formation of prostanoids. COX-2 is highly expressed in many cancers favoring cancer development by inhibiting apoptosis, and promoting cell proliferation, and angiogenesis (Sahin *et al*. 2009, Ghosh *et al*. 2010). HuR can also enhance the expression of tumor suppressor and angiogenesis inhibitor TSP1. However, when MCF7 breast cancer cells overexpress the MCT-1 (multiple copies in T cell lymphoma 1) oncogene, the association of HuR with TSP1 mRNA is diminished (Mazan-Mamczarz *et al*. 2008a), suggesting a dual role for HuR: When cancer develops, the association of HuR with its target mRNAs alters, favoring cancer development.

Immune system scrutiny leads to elimination of harmful material from the body such as tumor cells, so tumor cells must create some mechanism to avoid recognition by the immune cells. HuR raises MKP-1 levels in immune cells by promoting translation, thus causing suppression of immune function (Barbisan *et al*. 2009, Lim *et al*. 2009). Transforming growth factor β (TGF-β) can suppress the development of early-stage tumors, but it promotes proliferation, invasiveness, and metastasis in late-stage tumors (Watnick *et al*. 2003, Stoppoloni *et al*. 2008). Some studies have linked the tumorigenic effect of TGF-β to the fact that it enables tumor cells to evade immune recognition (Zabrenetzky *et al*. 1994, Keyse 2008). HuR binds and regulates TGF-β expression post-transcriptionally in malignant brain tumors (Nabors *et al*. 2001). Additionally COX-2, a target for HuR regulation, can modify the immunological response by altering the expression of the immunoactive cytokines which aid tumor cells in

escaping immune system elimination (Ghosh *et al*. 2010).

To be able to invade adjacent tissue or metastasize to distant tissues, tumor cells need to acquire the ability to change their interaction with their local environment. HuR can enhance cell motility by improving the expression of proteins related to degradation of extracellular matrix (matrix metalloproteinase 9 (MMP-9), uPA, and uPAR) and by enhancing the expression of Snail, a protein related to epithelial-to-mesenchymal transition, EMT (Akool *et al*. 2003, Huwiler *et al*. 2003, Tran *et al*. 2003, Dong *et al*. 2007).

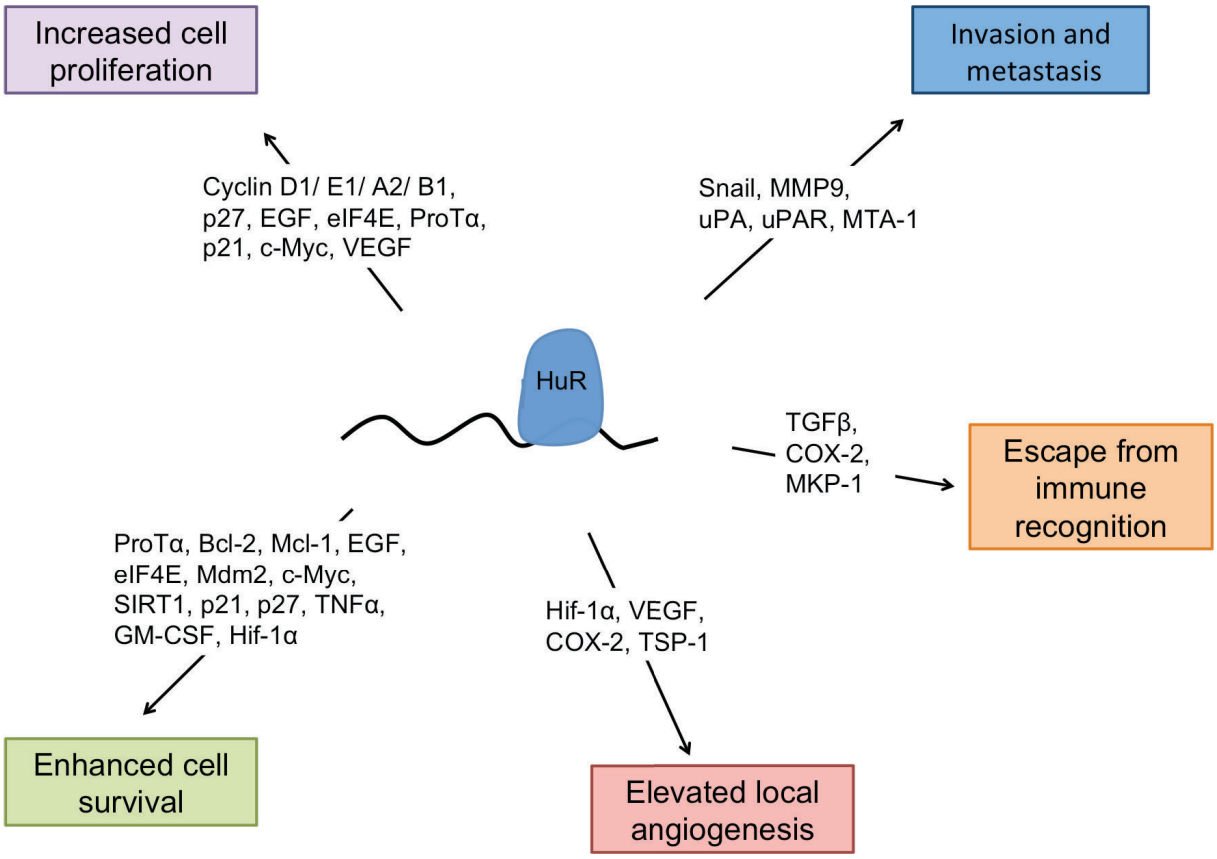


Figure 5. HuR participates in cancer development via regulation of its target transcripts.

3.2 HuR expression in cancer

The first hint of the role of Hu proteins in cancer was reported in small cell lung cancer where a higher titer of anti-Hu antibody was associated with a paraneoplastic syndrome (Dalmau *et al*. 1990). One of the first human cancer tissue studies was performed by Nabors *et al.* (2001), who showed that tumors of the central nervous system express HuR. All brain tumor tissues studied expressed HuR; the strong nuclear HuR protein expression was, however, limited to high-grade malignancies (glioblastoma multiforme and medulloblastoma). The importance of cytoplasmic HuR expression in cancer biology was first indicated in mice where chemically

induced lung tumors showed elevated cytoplasmic HuR expression (Blaxall *et al*. 2000). Today, the role of HuR in cancer development and its association with clinicopathological parameters have been studied with cell lines and clinical cancer specimens in several cancers. Importantly, HuR mRNA levels do not change noticeably between normal and cancer tissue (Brennan *et al*. 2009, Guo *et al*. 2009), but the intracellular localization of HuR protein seems important.

In colon carcinoma, the subcellular location of HuR within the tumor cells varies from that in normal colon cells (Lopez de Silanes *et al*. 2003, Young *et al*. 2009). The relative cytoplasmic abundance of HuR is lowest in the normal mucosa, moderately higher in adenomas, and highest in carcinomas. In normal mucosa, HuR expression is strongest at the base of the crypt and decreases towards the top of the crypt. HuR expression associates strongly with cellular proliferation (Lopez de Silanes *et al*. 2003). This suggests a possible role for HuR in colon carcinogenesis to upregulate the expression of proliferative cells. In nude mice, colon carcinoma cells that overexpress HuR develop larger tumors (Lopez de Silanes *et al*. 2003). Conversely, HuR inhibition with siRNAs reduces the growth of tumors in nude mice (Lopez de Silanes *et al*. 2003).

Cytoplasmic HuR positivity has been found in 53% of colorectal adenocarcinoma cases and the expression of cytoplasmic HuR associated with increased COX-2 expression as well as high tumor stage (Denkert *et al*. 2006). A large tissue microarray analysis of colorectal adenocarcinoma cases showed that along with overall TNM stage, high total HuR expression within the tumors was an independent marker for poor prognosis (Yoo *et al*. 2009). Neither cytoplasmic nor nuclear expression of HuR showed statistically significant impact on overall survival. However, patients with a high nuclear-to-cytoplasmic ratio of HuR expression show improved survival (Yoo *et al*. 2009). Cytoplasmic HuR expression has been studied also in normal colorectal mucosa, adenomas, adenocarcinomas from familial adenomatous polyposis patients, and sporadic colorectal carcinoma. Cytoplasmic HuR expression was increased in familial adenomatous polyposis adenocarcinomas and sporadic carcinomas, and also here was associated with elevated COX-2 expression (Brosens *et al*. 2007).

In gastric adenocarcinoma, cytoplasmic HuR expression associates with high COX-2 expression and with reduced survival from the disease, whereas nuclear HuR positivity does not (Mrena *et al*. 2005). HuR expression has been aberrantly elevated in gastric tumors when compared to nontumorigenic tissue. Additionally, nuclear factor κB enhances HuR transcription and PI3K/AKT signaling to activate HuR and lead to progression of human gastric cancer (Kang *et al*. 2008).

In ovarian carcinomas, nuclear as well as cytoplasmic HuR expression is significantly higher than in borderline tumors (tumors of low malignant potential) or in healthy ovaries. Cytoplasmic HuR expression associates significantly with increased COX-2 expression, high histologic grade, and mitotic activity. Cytoplasmic HuR expression is an independent marker of reduced survival in ovarian cancers (Denkert *et al*. 2004a). In serous type ovarian cancer, cytoplasmic HuR expression associates with high COX-2 expression, high tumor grade, and

reduced survival (Erkinheimo *et al.* 2003); in mucinous ovarian carcinoma, cytoplasmic HuR expression correlates with increased COX-2 expression (Erkinheimo *et al.* 2005). Additionally, cytoplasmic HuR expression in ovarian cancer has been associated with reduced survival and elevated β-tubulin levels (Raspaglio *et al.* 2010). Interestingly, in one study (Yi *et al.* 2009), nuclear HuR expression associates with the invasive type of the cancer, with high grade, large residual disease, and poor disease-free survival. Nuclear HuR expression is an independent prognostic factor of poor survival. These data indicate that nuclear HuR also may play a role in ovarian carcinogenesis, but the function of HuR in the nucleus needs further clarification.

In normal prostate tissue, HuR expression is predominantly nuclear, while in the cancer cases the cytoplasmic HuR expression is elevated (Niesporek *et al.* 2008). Similarly, in another study involving clinical specimens from normal-looking prostate epithelium, prostate tissue with atrophy, high-grade prostatic epithelium, and prostate carcinoma, the cytoplasmic HuR expression increased towards a more malignant phenotype (Barbisan *et al.* 2009). Cytoplasmic expression of HuR is elevated in prostate cancer and this is linked to altered expression of COX-2 as well as to adverse patient prognosis, suggesting that cytoplasmic HuR expression associates with prostate carcinogenesis and development (Niesporek *et al.* 2008, Barbisan *et al.* 2009).

Interestingly, in pancreatic cancer cases, high cytoplasmic HuR expression is found to be beneficial for patients: HuR overexpression has sensitized pancreatic cancer cells to gemcitabine at least in part by associating with dCK mRNA, a deoxy-cytidine kinase that metabolizes and activates gemcitabine in the cells. In pancreatic adenocarcinoma patients treated with adjuvant gemcitabine-based radiochemotherapy or chemotherapy, those patients with low cytoplasmic HuR expression had a seven-fold greater mortality than did patients with high cytoplasmic HuR expression (Costantino *et al.* 2009). HuR seems to be a key mediator of gemcitabine efficacy by regulating dCK abundance in pancreatic cancer cells. However, in the absence of a comparable group of patients receiving no gemcitabine, the predictive value of HuR cannot be truly evaluated (Costantino *et al.* 2009, Marechal *et al.* 2009). Recently, patients with high cytoplasmic HuR expression showed better overall survival than did patients with low cytoplasmic HuR expression; however, in the same patients, high cytoplasmic HuR expression associated with high tumor T-stage (Richards *et al.* 2010). Increased HuR levels and activity may facilitate a more efficient drug response, but conversely, increased HuR levels may also promote tumor cell survival by facilitating protein synthesis or stabilizing mRNAs beneficial for cancer development or both.

HuR expression has also been studied in Merkel cell carcinoma (MCC), a rare neuroendocrine carcinoma of the skin. There, primary MCCs and their lymph node metastasis express cytoplasmic HuR, while in contrast the non-neoplastic skin does not (Koljonen *et al.* 2008). Cytoplasmic expression of HuR may contribute to the carcinogenesis of MCC. Ewing sarcoma is a malignant round-cell tumor, and these cancer cells are found in bone or in soft tissue, most often in the pelvis, femur, humerus, and ribs. In Ewing sarcoma, 17% of the cases showed nuclear HuR positivity, but no cytoplasmic HuR expression was detectable (Do *et al.*

2008). In mesothelioma, a cancer primarily caused by exposure to asbestos, overall survival is significantly shortened in patients who express cytoplasmic HuR (Stoppoloni *et al*. 2008), and in renal cell carcinoma (RCC), cytoplasmic HuR expression associates with tumor stage, and reduced survival (Ronkainen *et al*. 2010). That HuR regulates COX-2 expression in RCC cells may be a plausible mechanism of the HuR-mediated aggressive behavior of RCC. HuR is expressed in a variety of cancers, showing an association with reduced survival, which suggests that HuR plays a role in the development of several cancers.

3.3 HuR in mice

In vitro HuR regulates genes involved in cellular processes such as tumorigenesis, cell cycle control, inflammation, cell stress response, and apoptosis (Brennan *et al*. 2001, Dixon *et al*. 2001, Gorospe 2003, Sengupta *et al*. 2003, Abdelmohsen *et al*. 2007a). The role of HuR *in vivo* is, however, still quite unclear. HuR expression can already be detected at embryonic day (E) 8.5, and from then on it is expressed at every stage of embryonic development, and up to adulthood (Gouble *et al*. 2000). Overexpression of HuR in transgenic mice leads to a defect in male gamete functioning, and this suggests a role for HuR in spermatogenesis (Levadoux-Martin *et al*. 2003). Heterozygous *Elavl1*+/- mice appear phenotypically normal and are able to reproduce (Ghosh *et al*. 2009, Katsanou *et al*. 2009), but *Elavl1*-/- mice die in utero. This embryonic lethality is due to their defects in extraembryonic placental development, meaning that HuR expression is required for labyrinth branching morphogenesis (Katsanou *et al*. 2009). The epiblast-specific deletion of HuR also leads to an embryonic lethality, at the latest on E19.5. Embryos have major defects in skeletal and spleen development, and dysmorphologies in the lungs (Katsanou *et al*. 2009), suggesting a multifaceted role for HuR during embryonic development. Postnatal global deletion of HuR causes atrophy of hematopoietic organs, extensive loss of intestinal villi, obstructive enterocolitis, and death of the mice within 10 days (Ghosh *et al*. 2009). Depletion of HuR leads to a decline in actively proliferating progenitor cells, leaving quiescent stem cells and differentiated cells unaffected. Expression of p53 and its downstream effectors are upregulated after HuR deletion, suggesting that HuR controls p53 levels in progenitor cells, thus promoting survival (Ghosh *et al*. 2009).

3.4 HuR in treatment of cancer

Drug-resistance is a major problem in cancer treatment. A recent discovery is that several cancer drugs can work as activators for HuR, ones such as gemcitabine, paclitaxel, and tamoxifen. In estrogen receptor-expressing breast cancer cells, tamoxifen treatment causes inhibition of ER activity, but at the same time causes activation of JNK, leading to elevated cytoplasmic HuR expression and an HuR-mediated increase in stability of mRNAs important in drug-resistance and enhancement of cell survival (Hostetter *et al*. 2008). Similarly, in hormone-refractory prostate carcinoma cells, reduced miR-34 levels in the cells raise HuR

expression and enhance SIRT1 and Bcl-2 expression, leading to enhanced paclitaxel-resistance (Kojima *et al*. 2010). In clinical ovarian cancer specimens, a strong correlation between cytoplasmic HuR expression and class III β-tubulin appears, and in ovarian cancer cells the cell responsiveness to cisplatin, paclitaxel, and tiocolchicine is restored by inhibiting class III beta-tubulin, which is also shown to be an HuR target (Raspaglio *et al*. 2010). Additionally, HuR binds the 3'UTR of p21 after irradiation and enhances its stability, suggesting that HuR can enhance p21 expression during radiotherapy, thus causing increased treatment-resistance by cancer cells. These examples all suggest that HuR regulation in cancer cells is important in the development of drug resistance. However, in pancreatic cancer cells, HuR overexpression can sensitize cells to gemcitabine treatment by raising the protein levels of deoxycytidine kinase, the enzyme that metabolizes and activates gemcitabine (Costantino *et al*. 2009); actually, patients with high cytoplasmic HuR expression survive better than do patients expressing low cytoplasmic HuR levels.

The low molecular-weight inhibitors of HuR thus far developed have interfered with HuR-RNA binding, HuR trafficking, cytokine expression, and T cell activation, and are antitumorigenic, antiangiogenic, and anti-inflammatory (Meisner *et al*. 2007). However, it remains to be seen whether these compounds will make it into the clinics.

AIMS OF THE STUDY

This study was undertaken to elucidate the role of HuR in breast carcinogenesis.

The specific aims of the projects were to:

I Investigate the localization and clinicopathological significance of HuR protein expression in breast cancer.

II Explore the association of HuR with clinicopathological variables and its prognostic significance in hereditary breast cancers.

III Elucidate the localization and significance of HuR expression in ductal in situ carcinoma and study the role of HuR in breast carcinogenesis *in vitro*.

MATERIALS AND METHODS

For each method, the relevant original publications are referred to by their Roman numerals.

1. Patient material (I, II, III)

This study utilized paraffin-embedded tissue material from women with sporadic invasive ductal breast carcinoma (I), a family history of breast cancer (II), DCIS (III), ADH (III), and from women with healthy tissue (III) from breast reduction surgeries. Detailed description of the patient materials is in the original publications.

The study projects were performed with the informed consent of patients as well as permission from the Ethics Committee of the Helsinki University Central Hospital and from the Ministry of Social Affairs and Health in Finland.

2. Immunohistochemistry (I, II, III)

HuR protein expression was analyzed in the breast tissue specimens by immunohistochemical staining. From the formalin-fixed and paraffin-embedded breast tissues, 5-µm sections were cut and processed for immunohistochemistry.

The immunostaining protocol of human specimens for HuR was carried out as follows: Specimens were deparaffinized and antigen retrieved by use of a microwave oven (4 x 5 min in 700 W in 0.01 M Na-citrate buffer at pH 6.0 for sporadic and familial breast cancer specimens and Tris-HCl at pH 8.5 for DCIS, ADH, and normal breast specimens). Tissues were immersed in 0.6% hydrogen peroxide in methanol for 30 min to prevent endogenous peroxidase activity and in blocking solution (1.5:100 normal horse serum in PBS) for 15 min to block unspecific binding sites. Immunostaining for HuR was performed with monoclonal antibody 19F12 (a kind gift of Dr. Henry Furneaux, University of Connecticut Health Center, Farmington, CT, USA), which was raised against a unique peptide from the NH_2 terminus of HuR, at a dilution of 1:20,000 (0.5 µg/ml; sporadic breast cancer specimens) or 1:10,000 (1.0 µg/ml; familial breast cancer, DCIS, ADH, and normal breast specimens) in PBS containing 0.1% sodium azide and 0.5% BSA. Additionally, DCIS material was immunohistochemically stained with HuR 3A2 antibody at a dilution of 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The specimens were incubated with antibodies at room temperature overnight. The sections were then treated with biotinylated horse antimouse immunoglobulin (1:200; Vector Laboratories Inc., Burlingame, CA, USA) and avidin-biotin peroxidase complex (Vectastain ABComplex, Vector). The peroxidase staining was visualized with 3-amino-9-ethylcarbazole (Sigma-Aldrich, Inc., St. Louis, MO, USA), and the sections were counterstained with Mayer's hematoxylin. In sporadic breast cancer material, to confirm the

specificity of the staining, a subset of the specimens (n = 7) was restained with and without the antigenic peptide for 19F12 (10 μg/ml; Clongene LLC, Hartford, CT, USA) for 1 h at room temperature before the staining procedure. To control the quality of HuR staining, each staining set contained predetermined breast or colon carcinoma specimens.

For mouse mammary gland tissue samples, the protocol was identical except that the specimens were pretreated with Vector M.O.M. The Basic Immunodetection Kit (Vector), which blocks endogenous immunoglobulins in mouse tissue and enables the use of mouse monoclonal antibody. The 19F12 HuR antibody was used at a dilution of 1:20,000 (0.5 μg/ml).

3. Evaluation of immunostaining (I, II, III)

HuR immunoreactivity was scored for each breast specimen independently and in a blinded manner by two investigators (M. H. and A. R.). HuR immunostaining was scored based on cytoplasm-negative, low-intensity cytoplasmic HuR staining present (visible at 100x or higher magnification), high intensity cytoplasmic HuR staining present (visible at 50x or lower magnification). Each staining set of 20 specimens contained two predetermined colon or breast carcinoma control slides, one of which contained only nuclear staining in the tumor cells and another one with additional cytoplasmic immunopositivity. All specimens with discordant scores were reevaluated by the two investigators using a multiheaded microscope, and the consensus score served for further analyses.

4. Tissue cultures (III)

The 184B5Me human mammary epithelial cell line was obtained by the transfecting parental 184B5 cell line with the erbB2 oncogene and selecting colonies that grew anchorage-independent (a kind gift of Dr Martha Stampfer, University of California, Berkeley, CA, USA and based on Novak *et al*. 2009). 184B5Me cells were cultured in MCDB 170 growth medium (US Biological, Swampscott, MA, USA) supplemented with 5 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 70 μg/ml bovine pituitary extract, 5 μg/ml transferrin, 10^{-5}M isoproterenol, 50 μg/ml amphotericin B, and 50 μg/ml gentamicin (all reagents from Sigma-Aldrich Inc.). 293FT and MCF7 cells were cultured in DMEM medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. All cells were cultured at 37 °C at 5% CO_2.

5. Production of HuR-silencing (III) and -overexpressing constructs (unpublished data)

To silence HuR expression in the cells, a lentiviral HuR shRNA construct was developed. Short hairpin RNAs (shRNA) are formed from siRNAs with a stem-loop hairpin structure; shRNA is inserted into an expression vector and transfected into cells where it can form functional siRNAs. For planning HuR shRNA oligos we used Applied Biosystem's Ambion siRNA Converter software (www.ambion.com/techlib/misc/psilencer_converter.html). Our best working HuR siRNA sequences (target sequence: 5'-AACATGACCCAGGATGAGTTA-3') were entered into the software with loop-structure (TTCAAGAGA) and plasmid (p*Silencer*), after which the Ambion siRNA converter produces the sequences for HuR shRNA oligos. The control shRNA construct expressed a nontargeted scrambled sequence of an HuR shRNA oligo sequence (SCR shRNA). The oligos for HuR and SCR shRNA are presented in Table 4. The shRNA oligos were annealed with PCR (95 °C for 4 min, 70 °C for 10 min, 0.3 °C/sec until the temperature reached 4 °C) and ligated into a pENTR-H1-BgH entry plasmid. Ambion siRNA Converter software generates BamHI (5'G•GATCC3') and HindIII (5'A•AGCTT3') restriction sites to the ends of the oligos, which enables direct ligation (15 °C overnight) to the entry plasmid.

To produce the shRNA sequence-containing entry plasmid, Stbl3 One Shot Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA, USA) cells were transformed by heat shock (45 sec at 42 °C) and plated on LB agar supplemented with Kanamycin (50 μg/mL, Sigma-Aldrich) and incubated at 37 °C overnight. Several colonies formed were chosen for cultivation following plasmid purification by the Macherey-Nagel Plasmid DNA Purification Kit (Macherey-Nagel GmbH & Co, Düren, Germany). Correctness of the isolated plasmids was checked by test restrictions and sequencing. After confirming the right products, the shRNA sequence was transferred to the end plasmid (pDSL_hpUGIH) with LR recombination. The LR recombination reaction (entry clone 75 ng, destination clone 150 ng, LR clonase enzyme, 5x LR clonase TM reaction buffer, TE buffer pH 8.0, total volume 5 μl per reaction, Invitrogen) was incubated at room temperature overnight, after which proteinase K was added to inactivate endogenous nucleases. Stbl3 One Shot Chemically Competent *E. coli* (Invitrogen) cells were used again for transformation as above. The quality of the constructs was defined before continuing to virus particle production.

To produce HuR overexpressing construct, the HuR coding sequence was copied from the pZeoSV2-HuR (sense) plasmid (kind gift of Dr. Myriam Gorospe, NIH, Baltimore, MD, USA) by PCR (98 °C for 30 sec following 30 cycles of 98 °C for 5 sec, 52 °C for 30 sec, and 72 °C for 15 sec, after which 5 min at 72 °C and held at 4 °C; Table 4). The molar ratio 1.5:1 was used for directional TOPO® cloning to introduce HuR cDNA into the pENTR™/D-TOPO® entry-vector, and transformed to the TOP10 One Shot Chemically Competent *E. coli* cells (Invitrogen) by heat shock (30 sec at 42 °C). After selection and validation of an appropriate clone, LR recombination was performed as above. The destination vector was pLenti6/V5 (Invitrogen).

Table 4. Primers for HuR silencing and overexpression constructs.

Construct	**Primer sequence**
HuR shRNA	
Forward	5'-GATCCG CATGACCCAGGATGAGTTA TTCAAGAGA TAACTCATCCTGGGTCATG TTTTTTGGAAA-3'
Reverse	5'-AGCTTTTCCAAAAAA CATGACCCAGGATGAGTTA TCTCTTGAA TAACTCATCCTGGGTCATG CG-3'
SCR shRNA	
Forward	5'-GATCC GAACTTCGCGATGGATACA TTCAAGAGA TGTATCCATCGCGAAGTTC TTTTTTGGAAA-3'
Reverse	5'-AGCTTTTCCAAAAAA GAACTTCGCGATGGATACA TCTCTTGAA TGTATCCATCGCGAAGTTC G-3'
HuR	
Forward	5' CACCGACTCACTATAGGGA 3'
Reverse	5' TTTGTGGGACTTGTTGGT 3'

6. Virus particle production (III)

The lentiviral particles were produced by transfecting 293FT cells with Lipofectamine 2000 (Invitrogen) containing a mixture of three plasmids: an HuR expression-modifying vector or its control vector (20 μg), a packaging plasmid (CMVdelta8.9, 15 μg), and a VSVg-coding envelope vector (pHCMV-G, 10 μg). Transfections were performed as follows: in tube A, 45 μg of plasmid DNA mix and in tube B, 112 μl of Lipofectamine 2000 was added to 2800 μl of plain cell culture media. These tubes were incubated separately for 5 minutes at RT, after which they were combined, mixed by flicking of the tube, and incubated for 20 minutes at RT. Then 19 ml complete growth media without antibiotics was added onto the plasmid DNA-Lipofectamine media, and mixed by inverting the tube. Eight ml of this mix was added to 293FT cells (10 cm ∅) and incubated at 37 °C in 5% CO_2 for 5 hours, after which the complete growth media was changed. Transfected 293FT cells were cultured for 72 hours at 37 °C in 5% CO_2, after which the supernatant was collected, sterile-filtered, and used directly on cell infections or stored at -80 °C.

7. Cell infection with virus particles (III)

A spin-down method was used to infect cells. Cells were seeded on a 6-well plate, and on the following day, virus infection was performed. Briefly, cells were incubated with virus particle media containing 8 μg/ml polybrene for 10 minutes at 37 °C in 5% CO_2, centrifuged (Multifuge 3 S-R, Sorvall/Heraeus) at 2500 rpm for 30 minutes at RT, and incubated again at 37 °C in 5% CO_2 for 2 hours, after which the complete growth medium was changed on the cells. Cells were cultured at 37 °C in 5% CO_2 for 72 hours, after which selection was started

with Hygromycin B (200 μg/ml for HuR shRNA, Invitrogen) or Blasticidin (5 μg/ml for HuR cDNA, Invitrogen).

8. Cell transfections (III)

Two HuR siRNA were used for 184B5Me breast epithelial cell transfections. The first HuR siRNA (Marked: siHuR1, target sequence: 5'-AACATGACCCAGGATGAGTTA-3', Dharmacon, Thermo Scientific, Lafayette, CO, USA) sequence is the same that we used to produce the HuR shRNA construct. The second siRNA against HuR, ON-TARGET plus SMARTpool (Marked: siHuR2, Dharmacon, Thermo Scientific), is a siRNA pool containing four separate siRNA sequences against HuR, but not the siHuR1 sequence. The control siRNA was ON-TARGET plus Non-targeting Pool (Dharmacon, Thermo Scientific).

Cells were seeded on 6-well plates in antibiotic-free media. On the following day, cells were treated with 100 nM siRNA targeting HuR or with control siRNA using Lipofectamine 2000 (Invitrogen), double the amount recommended in the manufacturer's instructions. Cells were incubated with siRNAs for 4 hours at 37 °C in 5% CO_2, after which the complete growth media was changed. Incubation was continued for 72 hours at 37 °C, and mRNA and proteins were isolated.

9. HuR immunofluorescence (III)

184B5Me cells were fixed with 4% paraformaldehyde for 20 minutes at RT, permeabilized with 0.5% NP40-PBS for 5 minutes at RT, and blocked with 3% bovine serum albumine (BSA) in Tris-buffered saline (TBS) for 20 minutes at RT. HuR 19F12 primary antibody (Santa Cruz Biotechnology) was in a dilution of 1:200 in 3% BSA-TBS and incubated at 37 °C for 75 minutes. Alexa-fluor® goat anti-mouse 546 IgG secondary antibody (Invitrogen) was used at a dilution of 1:200 at 37 °C for 45 minutes, and cells were mounted with the Vectashield Mounting Medium (Vector).

10. Protein extractions and Western blot analysis (I, III)

Mouse mammary glands were dissected from CD1 wild-type mice and from CD1 COX-2 transgenic mice (Liu *et al*. 2001) and homogenized in an extraction buffer containing 1% Tween 20, following Western blot analysis using 60 μg of protein. A monoclonal 19F12 HuR antibody was used at a 1:10,000 dilution, and COX-2 was detected by a monoclonal antibody at a 1:5,000 dilution. Loading was controlled with ß-actin antibody (1:10,000 dilution; Santa Cruz Biotechnology).

Total proteins were isolated with a radioimmuno precipitation assay (RIPA) –buffer (150 mM NaCl, 1% NP-40, 1% DOC, 0.1% SDS, 50 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) with proteinase inhibitors or a NucleoSpin® RNA/protein kit (Macherey-Nagel GmbH & Co) using 1x Laemmli buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.002% bromphenolblue). Cytoplasmic and nuclear fractions were prepared from cell cultures by use of an NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL, USA). Protein concentrations were measured in the lysates with a spectrophotometer (Multiscan Ascent, Thermo Labsystems). Proteins were separated on 12% SDS-PAGE and transferred onto Hybond-C extra nitrocellulose membranes (Amersham, Buckinghamshire, UK). Nonspecific binding was blocked by incubating membranes in a 5% non-fat dry milk TBS-NP40 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40) solution overnight at 4 °C. Membranes were incubated in primary and secondary antibodies for one hour at room temperature. Primary antibodies were mouse monoclonal rabbit polyclonal ATF-2 (1:100), mouse monoclonal CTGF (1:50), HuR 3A2 (1:2000), rabbit polyclonal Rab31 (1:50), rabbit polyclonal anti-β-tubulin (1:500), goat polyclonal anti-β-actin (1:1000, all these antibodies from Santa Cruz Biotechnology), rabbit polyclonal HER-2 (1:1000, Upstate, Lake Placid, NY, USA), and rabbit polyclonal anti-lamin A/C (1:500, Cell Signaling Technology, Inc., Danvers, MA, USA). Secondary antibodies used were goat anti-mouse (1:2000 for HuR, 1:500 for CTGF, Pierce Biotechnology, Rockford, IL, USA), goat anti-rabbit (1:500, Pierce), and donkey anti-goat (1:2000, Santa Cruz Biotechnology).

11. RNA isolation and Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR, III)

RNA was isolated with the NucleoSpin® RNA/protein kit (Macherey-Nagel GmbH & Co). One microgram of RNA was converted to cDNA with M-MLV (moloney-murine leukemia virus) reverse transcriptase (Promega, Madison, WI, USA) and by random primers (Invitrogen) for 1 hour at 37 °C in a volume of 50 µl. The reaction was stopped by heat inactivation (95 °C for 3 minutes) and filled with sterile water to a final volume of 200 µl. Quantitative RT-PCR gene expression assays were performed with the GeneAmp 7500 Sequence detection system according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Briefly, a PCR reaction-master mix was prepared for each reaction: 1.25 µl of 20x target assay mix (target gene), 1.25 µl of endogenous control, 8 µl of RNAse-free water, and 12.5 µl of 2x TaqMan Universal PCR MasterMix. Then 23 µl of PCR master mix and 2 µl of cDNA template were pipeted per reaction (total volume 25 µl). PCR cycling conditions were as follows: 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles of 95 °C denaturation for 15 seconds and 60 °C annealing for 1 minute. ATF2 (Hs00153179_m1), COX-2 (Hs00153133_m1), CTGF (Hs00170014_m1), DPYD (Hs01115751_m1), E-cadherin (Hs00170423_m1), ErbB2 (Hs01001580_m1), GRB14 (Hs00610306_m1), HuR (Hs00171309_m1), MTUS1 (Hs00826834), p21 (Hs00355782_m1), p53 (Hs00153349_m1), PRKD1 (Hs01554327_m1), Rab27a (Hs00608302_m1), Rab31 (Hs00924848_m1), Snail (Hs00195591_m1), Slug (Hs00161904_m1), TFPI2

(Hs00197918_m1), Twist (Hs00361186_m1), VCAN (Hs00171642_m1), Vimentin (Hs00185584_m1), Zeb1 (Hs00170423_m1), and Zeb2 (Hs00207691_m1) TaqMan® gene expression array primers (Applied Biosystems) were used, with human 18S rRNA as an endogenous control. Each sample had three replicates, and data were analyzed by the delta-delta method to compare relative expression results (2-ddCt).

12. mRNP immunoprecipitation for HuR (III)

HuR binding to its possible mRNA targets was studied in 184B5Me breast epithelial and MCF7 breast cancer cells. Endogenous RNA protein complexes were immunoprecipitated (IP) from the cell lysates with a 50% (v/v) suspension of Protein A-Sepharose beads (Sigma-Aldrich) precoated with 30 µg of either anti-HuR antibody (Santa Cruz Biotechnology) or IgG1 control (BD Pharmingen). The beads were washed with NT2 buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.05% Nonidet P-40. For the analysis of RNAs in the IP, 100 µl of NT2 buffer supplemented with 5 µl DNase I (2U/µl) were added to the beads, incubated at 37 °C for 10 min, washed with NT2 buffer, and further incubated with 100 µl of NT2 buffer containing 5 µl of Proteinase K (10 mg/ml) and 1 µl of 10% SDS at 55 °C for 30 min. RNA was isolated and precipitated in the presence of glycoblue with acid phenol-$CHCl_3$, sodium acetate, and ethanol.

The mRNA levels of ten target genes were studied in MCF-7 and 184B5Me cell lines using PCR. GAPDH served as an endogenous control, and results were normalized to it. PTMA and EIF4EBP2 were positive controls. Primers for the qRT-PCR are presented in Table 5.

Table 5. Primers to study HuR binding with its possible target mRNAs by qRT-PCR.

Gene name	Forward primer	Reverse primer
ATF-2	ACCATGCCTGTTGCTATTCC	CCTGGAACACTAGGCACCAT
CTGF	TCCCACCCAATTCAAAACAT	TGCTCCTAAAGCCACACCTT
DPYD	CGGGGAGTCGTGATTGTACT	GTTCCATCTCCTCAGGGACA
EIF4EBP2	CTTCCCTTTGTCTCCCATGA	TCACACAGGACTGCCTCAAG
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
GRB14	CTCTGCCACAAACATGGCTA	CCCGTACCAAGAAAACTCCA
MTUS1	GGACAACAACACAGCATTGG	GGAAAGCTGCCTTGAGATTG
PRKD1	GCCATGACCCTACCTCTGAA	TGGGGACGAATCTGAAAGTC
PTMA	CCAACCCAAACCATGAGAA	GGTCACACCACAAGTAAAGTCAG
RAB27A	ATCACAACAGTGGGCATTGA	CCCTGCTGTGTCCCATAACT
RAB31	CGCAAAAGTCAGGAAAGAGG	GTGTTTCCCAGCCTTCACAT
TFPI2	CGCACCAAAGAAAATTCCAT	ATTGTCATTCCCTCCACAGC
VCAN	TGTTCCTCCCACTACCCTTG	CTTCCACAGTGGGTGGTCTT

13. Cell growth measured by CellTiterBlue (III)

To measure cell growth on two-dimensional cultures, the fluorometric method CellTiterBlue® Cell Viability Assay (Promega Corporation, Madison, WI, USA) was the choice. In the assay, the viable cells are able to reduce the indicator dye resazurin to resorufin, which is highly fluorescent. Cells were seeded on 96-well plates ($1x10^3$ cells / well in 100 µl media). On the measuring day, 20 µl per well of resazurin-containing reagent was added to cells then incubated at 37 °C in 5% CO_2 for 2 hours, and the amount of fluorescent signal was measured with the FLUORstar OPTIMA Microplate Reader (BMG LABTECH Inc., Durham, NC, USA). Cell growth was measured on days 1, 4, and 7 after plating.

14. Cell growth measured by cell counting (unpublished data)

Cells ($1x10^4$ / well) were seeded in 2 ml of complete growth media in 6-well plates, and the number of cells was counted on days 1, 4, and 7 after the plating with a Bürker counting chamber. The experiment was repeated twice, and three parallel samples were counted per cell line per experiment.

15. Anchorage-independent growth in soft agar (III)

Cells ($1x10^4$ / ml) were suspended in 1 ml of 0.25% agarose (GellyPhor, EuroClone Spa, Pero, Italy), layered over 1 ml of 0.5% agarose base layer and supplemented with 2 ml complete culture medium in a 6-well plate. After 10 days of culture in agarose, cells were fixed with methanol for 10 minutes and stained with Giemsa (1:20 dilution in H_2O, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Cell colonies were photographed with a Leica MZFLIII microscope with 3.2x enlargement and analyzed with ImageJ Software, taking 400 pixels as a cut-off for colonies. Colonies from the transient transfections were counted manually from the pictures.

16. Programmed cell death (anoikis) assay in methylcellulose (III)

Cells ($5x10^4$ / ml) were suspended in 3 ml of 0.5% methylcellulose (Sigma-Aldrich) complete growth media mix, and seeded on bacterial plates (35 mm in diameter, Greiner-Bio One GmbH, Frichkenhausen, Germany), three plates per cell line. Cells were cultured in 0.5% methylcellulose media mix for 9 days, after which formed cell clusters were counted.

17. Cell senescence assay (unpublished data)

Cell senescence was studied in the 184B5Me HuR-silenced cells and their controls with the Senescence Detection Kit (Biovision, Inc., Mountain View, CA, USA), which histochemically detects the expression of β-galactosidase (SA-β-Gal) activity. Cells were fixed with Fixative Solution for 10 to 15 minutes at RT, stained with the Staining Solution Mix overnight at 37 °C; development of blue color was detected with a light microscope.

18. Cell invasion assay using myoma organotypic culture (III)

We studied HuR-silenced cells and their controls' invasion ability in a myoma organotypic culture model (Nurmenniemi *et al.* 2009). Briefly, macroscopically homogeneous areas of uterine leiomyoma tissue were used to prepare myoma disks for the experiment. These disks were stored in 10% DMSO at -70 °C. To start the myoma organotypic culture, myoma disks were first equilibrated in the complete growth media of the cells at 4 °C with gentle rocking for 10 days. Equilibrated myoma discs were placed into Transwell inserts (6.5 mm in diameter; Corning Incorporated, Corning, NY, USA), and 5 x 10^5 HuR-silenced cells or control cells were seeded on the myoma disks in 50 µl of culture media. The cells were allowed to attach overnight, after which the disks removed from the Transwell inserts were transferred onto uncoated nylon disks resting on curved steel grids (3 mm x 12 mm x 15 mm) on 12-well plates in 1 ml of culture media. Myoma organotypic cultures were maintained at 37 °C for 10 days, and the media of the cultures were changed every 3 days. In the experiment, the disks prepared were from the same myoma.

At day 10, the organotypic cultures were fixed in 4% neutral-buffered formalin overnight, after which they were dehydrated, bisected, and embedded in paraffin. Mayer's hematoxylin and eosin, and pancytokeratin AE1/AE3 (1:150 dilution; Dako, Glostrup, Denmark) immunohistochemistry allowed identification of invaded cells.

19. *In vivo* mouse experiment (III)

HuR-silenced cell and control cell tumorigenity was studied in immunodeficient mice. This study was an outsourced service conducted at Pharmatest Services Ltd, Turku, Finland. The Hsd:Athymic Nude-Foxn1nu mouse strain (supplier: Harlan, Boxmeer, the Netherlands) was maintained at the Turku Science Park Biolaboratory (License: STO/1053 (8.9.1999), 1132/712-92, 1286/712-86). Five million cells in 120 µl cell culture media containing 30% Matrigel were orthotopically inoculated into the mammary fat pads of the female mice. Each group comprised 10 mice; HuR-silenced cells were inoculated into 18 mammary glands, but control (shSCR) cells into only 11 mammary glands because of an insufficient quantity of cells. The mice had free access to food and water. Tumor diameters were measured once a

week with a caliper and tumor volume was calculated according to the formula $(\pi/6)(d_1 x d_2)_{3/2}$, where d_1 and d_2 are perpendicular tumor diameters. The experiment continued for 12 weeks.

The experimental procedures were reviewed by the local Ethics Committee on Animal Experimentation at the University of Turku and approved by the local Provincial State Office of Western Finland (Licence: ESLH-2008-00340-Ym23).

20. Gene expression array (III)

GeneChip® Human Genome U133 plus 2.0 (Affymetrix Inc., Santa Clara, CA, USA) gene expression arrays were performed on the 184B5Me cells infected with the HuR-silencing construct and its control (n = 2 per cell line) at the Biomedicum Biochip Center (www.helsinki.fi/biochipcenter). Gene expression arrays were analyzed with CSC Chipster v1.3.0 software for DNA Microarray data analysis (http://nami.csc.fi). Microarray data were normalized by the gcrma (GeneChip Robust Multi-array Average) normalization method. The probes were re-annotated by use of alternative chip description file (CDF) environments (hs133phsentrezg (hgu133plus2)). In the re-annotation process, ambiguous probes that map to more than one position in the genome were discarded. This left 17 589 probe-sets for the further analyses. Data were further preprocessed, filtering genes according to their standard deviation, i.e. genes that show the lowest standard deviations are those that display little difference between HuR-silenced cells and their controls, and are filtered out. Cut-off point for the data to be filtered out was 95%. For statistical analyses, a two-group test was used with the settings empirical Bayes t-test, and the Benjamin-Hochberg false discovery rate for correcting multiple tests; the p-value cut-off was 0.01. For overexpressed transcripts, we selected only those induced by two-fold or higher, and for under-expressed those that were 0.5-fold or lower.

21. Cell network analysis (III)

The Moksiskaan (Laakso *et al*. 2010) computational platform allowed study of biological alterations in the cells after HuR silencing. The Moksiskaan integrates pathway, protein-protein interaction, drug target, and disease information from various databases. This information is compared to the given set of differentially expressed genes (DEGs) in order to describe their biological function and cross-links in the cells. Expression arrays were re-analyzed within the system to obtain the latest genome annotations and to make the data formats compatible with the tools. Genes with a fold change less than 1/1.5 were considered as down- and those > 1.5 as upregulated.

22. Statistical analysis (I, II, III)

The association between HuR staining and clinically relevant and prognostic variables was assessed by χ^2 test. Life tables were computed according to the Kaplan-Meier method, and survival curves were compared with the log-rank test. Distant disease-free survival was calculated from date of diagnosis to occurrence of metastases outside the regional area or to death from breast cancer. Overall survival was calculated from date of the diagnosis to death from breast cancer. Patients who died from intercurrent causes were censored at the date of death. Multivariate survival analyses were performed with the Cox proportional hazards model. A backward stepwise algorithm aids in the choice of the best combination of prognostic factors to explain the mortality in the study population. Hazard ratios are provided for each covariate. Cox regression involved a backward stepwise selection of variables, and a *p* of 0.05 was adopted as the limit for inclusion of a covariate. The non-parametric Mann-Whitney U-test served for the statistical analysis to compare the groups in cell growth, anchorage-independent growth, programmed cell death, and invasion assays. A p-value of $\leq$ 0.05 was considered statistically significant. All data are shown as means $\pm$ SEM. The data was analyzed with SPSS for Windows v12.0.1 (SPSS Inc., Chicago, IL, USA) or with SPSS 16.0 for Mac (SPSS Inc.).

RESULTS AND DISCUSSION

Results of the thesis projects are summarized and discussed here. Project-specific detailed presentations and discussions are in the original publications, referred to by their Roman numerals.

1. THE EXPRESSION AND PROGNOSTIC VALUE OF HUR IN THE BREAST (I, II, III).

When this thesis project began, the first hints had been found of the significance of HuR expression to carcinogenesis. Gliomas (high-grade brain tumors) expressed elevated levels of nuclear HuR (Nabors *et al*. 2001), and chemically induced mice lung tumors expressed elevated levels of cytoplasmic HuR (Blaxall *et al*. 2000). Additionally, a prognostic role for HuR in ovarian cancer had been suggested (Erkinheimo *et al*. 2003, Denkert *et al*. 2004a). We used a mouse mammary gland tumor model, where the human *COX-2* gene is overexpressed under the influence of a murine mammary tumor virus promoter (Liu *et al*. 2001), to study the expression of HuR in mouse mammary gland tumors. We found that formed breast tumors expressed higher levels of HuR than did healthy mammary gland tissue from wild-type control mice. Immunohistochemical analysis of these cancerous and healthy mice mammary gland tissues revealed that HuR expression had translocated to the cytoplasm in cancerous mammary glands, whereas in wild-type control mice its expression was mainly nuclear. Since cytoplasmic HuR mediates mRNA stabilization and translation of cancer-promoting genes (Abdelmohsen *et al*. 2010a), these results raised the question whether HuR is expressed in human breast cancer.

1.1 Expression of HuR in clinical specimens from the breast

We studied the expression and localization of HuR in healthy breast tissue, in preinvasive breast cancer lesions (ADH, DCIS) and in invasive sporadic ductal breast carcinoma and familial breast cancer specimens. ADH and DCIS associate with an increased risk for invasive breast cancer (RR of 4.0-5.0 for ADH and 10-12 for DCIS) (Tavassoli *et al*. 2003), and if left untreated, 14 to 75% of DCISs can proceed to invasive breast cancer (Mokbel *et al*. 2006). Although non-invasive, some genetic alterations have already occurred in ADH and DCIS that are comparable to changes in invasive breast cancer, such as loss of heterozygosity (Tavassoli *et al*. 2003). Currently, we have no method to separate from the preinvasive cases the ones, which will develop into an invasive breast cancer, which is the reason that surgery and clearance of axillary lymph nodes are performed as treatment for all the patients. Invasive ductal breast cancer (of no specific type) is the most common breast cancer type in women,

comprising 40 to 75% of all breast cancer cases (Tavassoli *et al*. 2003), and familial breast cancer comprises approximately 10% of all breast cancers (Venkitaraman 2002).

Mutations in two breast cancer susceptibility genes, *BRCA-1* and *BRCA-2*, predispose women to breast cancer. In *BRCA-1* mutation carriers, the cumulative risk for developing breast cancer before 70 years of age is 50 to 70%, and in *BRCA-2* mutation carriers, 40 to 50% (Venkitaraman 2009). Mutations in *BRCA* genes, however, explain only about 15% of all hereditary breast cancers, and an additional 10% of the cases are connected to the effects of other known cancer susceptibility genes such as *TP53, PTEN*, *ATM*, and *CHECK2*. The remaining hereditary breast cancers are possibly a consequence of a polygenic model in which polymorphism or mutations in a large number of genes, each conferring a small excess of risk, have a magnifying effect (Venkitaraman 2009).

Table 6. Cytoplasmic HuR expression in breast tissue specimens as detected by HuR 19F12 antibody.

Tissue	**N**	**Cytoplasmic HuR Expression, N (%)**	
		Negative	**Positive**
Healthy breast tissue	66	57 (71)	19 (29)
Atypical ductal hyperplasia	71	36 (51)	35 (49)
Ductal carcinoma *in situ*	74	39 (53)	35 (47)
Invasive ductal breast carcinoma	133	80 (60)	53 (40)
Familial breast cancer			
Non-BRCA1/2	525	318 (61)	207 (39)
BRCA-1	51	19 (37)	32 (63)
BRCA-2	47	18 (38)	29 (62)

Our results show that cytoplasmic HuR expression is already aberrant in ADH cases and remains elevated also in DCIS as well as in invasive cancers (Table 6). Cytoplasmic HuR expression was higher in healthy controls (29%) than has been generally reported, < 10% (Wang *et al*. 2000b, Lopez de Silanes *et al*. 2003, Yuan *et al*. 2010), but the cytoplasmic expression pattern of HuR was focal and localized to the luminal epithelial cells of the acinar and ductal structures. This may show up in healthy controls as a relatively high proportion of cytoplasmic HuR expression, since in our scoring system, the highest score of the tissue specimen was always noticed in the analysis. A recent report showed that HuR expression in the endometrium varies during the menstrual cycle, being highest from mid-proliferative phases (days 6-14) till mid-secretory phases (days 15-23) (Karipcin *et al*. 2010). Similarly, in the breast, HuR may be under endo-, para- or autocrine control, which may explain the focal expression of HuR in the cytoplasm of healthy controls. ADH, DCIS, and invasive cancer specimens expressed cytoplasmic HuR uniformly, and the expression was localized in neoplastic epithelial cells. Stromal tissue remained negative for HuR staining or showed only partial nuclear positivity. We did not, however, analyze systematically the expression of HuR

in the stroma, so drawing too strong conclusions as to HuR expression in the stromal tissue would be unwise.

1.2 Association of cytoplasmic HuR expression with clinicopathological parameters and survival

Study of the association of cytoplasmic HuR expression with clinicopathological parameters and survival should elucidate the potential of HuR to serve as a prognostic marker. In DCIS, cytoplasmic HuR expression associated with high grade, prostaglandin receptor negativity, and microinvasive or tumor cell-positive sentinel node findings. Cytoplasmic HuR expression was associated in these preinvasive specimens with a more aggressive form of the disease and suggests that HuR participates in tumor development towards invasive disease.

In invasive sporadic ductal breast carcinoma and familial non-BRCA1/2 cases, cytoplasmic HuR expression associated with p53 positivity, poorly differentiated tumors, and reduced disease survival. Additionally, in sporadic cases, large tumor size, and in familial non-BRCA1/2 cases, ductal type and hormone-receptor negativity associated significantly with cytoplasmic HuR expression. Most importantly, cytoplasmic HuR expression was an independent marker for reduced survival in both sporadic and familial non-BRCA1/2 breast cancers, meaning that cytoplasmic HuR expression alone can predict disease prognosis.

Additionally, we studied the prognostic capacity of cytoplasmic HuR expression in more detail by subgroup analysis. The data from these analyses show that high cytoplasmic HuR expression associated with reduced survival in the subgroups of older patient (age ≥ 50), small tumors (≤ 2 cm), poorly differentiated tumors, in lymph node-negative and -positive tumors, hormone-receptor-expressing tumors, and HER-2-negative tumors. This is an important finding, since especially in the subgroups of small and lymph node-negative tumors the need to forecast their individual course was immediate.

Our *BRCA-1* and *BRCA-2* mutated specimens showed the highest cytoplasmic HuR positivity (Table 6). These proteins play a crucial role in maintaining structural and numerical stability of chromosomes by participating in DNA repair and recombination, cell cycle control and transcription (Venkitaraman 2002, 2009). Although having similar effects in cells, BRCA-1 and BRCA-2 bear little similarity to each other or to proteins of known function (Venkitaraman 2009). Malfunctioning BRCA-1 or BRCA-2 protein leads to DNA damage in the cell, which causes increased cell stress. HuR is a cell stress-responsive protein and regulates many proteins involved in cellular response to DNA damage such as p21, p53, and cyclins A and B1 (Wang *et al*. 2000a, 2000b, Mazan-Mamczarz *et al*. 2003, Kim *et al*. 2010), which may explain the high cytoplasmic HuR expression found in BRCA-1 and -2 cases. Cytoplasmic HuR expression was associated only with p53 positivity in BRCA-1 cases. The reason for HuR's not associating with these clinicopathological parameters and showing no prognostic capacity may be that DNA damage causes multiple alterations in the cells, leading

to loss of a single factor. Interestingly, in *BRCA-1*-mutated cases, patients who expressed HuR in their cytoplasm survived better than did HuR cytoplasm-negative patients. It would be interesting to study, whether HuR expression is aberrant in the healthy tissue of *BRCA*-mutation carriers. However, since we had only tumor microarray specimens of these patients, we cannot draw any conclusions as to HuR expression even in adjacent tissue. HuR binds BRCA-1 mRNA and negatively regulates translation of the protein *in vitro* (Saunus *et al.* 2008). In this respect, cytoplasmic HuR expression may prevent the translation of mutated BRCA-1 and formation of malfunctioning DNA repair machinery (O'Donovan *et al.* 2010). However, because this result was not statistically significant, and knowing that HuR can also regulate so many other targets in the cytoplasm that are beneficial for cancer development, strong conclusions are impossible.

Our cytoplasmic HuR expression data for the breast specimens are in line with others' findings in breast carcinoma specimens (30%-75%) (Denkert *et al.* 2004b, Woo *et al.* 2009), as well in colon (53%-96%), gastric (40%), and ovarian (45%-70%) cancer cases (Erkinheimo *et al.* 2003, 2005, Denkert *et al.* 2004a, 2006, Mrena *et al.* 2005, Yi *et al.* 2009, Young *et al.* 2009), and HuR expression translocates from nuclear staining in healthy tissue to cytoplasmic staining in cancerous tissue (Lopez de Silanes *et al.* 2003, Koljonen *et al.* 2008, Niesporek *et al.* 2008, Barbisan *et al.* 2009, Young *et al.* 2009) Additionally, we found cytoplasmic HuR expression to associate with increased aggressiveness of the disease, and similarly an association has been commonly reported of cytoplasmic HuR expression with poorly differentiated tumors (Erkinheimo *et al.* 2003, 2005, Denkert *et al.* 2004a, 2004b, 2006, Niesporek *et al.* 2008, Stoppoloni *et al.* 2008, Barbisan *et al.* 2009). These results suggest that HuR translocation to the cytoplasm is important for breast cancer development and plausibly could serve as a prognostic marker in clinics.

2. THE ROLE OF HUR IN BREAST CARCINOGENESIS (III)

Clinical material results suggest a role for HuR in breast carcinogenesis. To explore the mechanisms by which HuR mediates carcinogenesis at its pre-invasive stage towards invasive breast cancer, we chose the 184B5Me breast epithelial cell line. This cell line was created by transfecting 184B5 breast epithelial cells with the erbB2 oncogene and selecting for anchorage-independent growth (Novak *et al.* 2009). We modulated HuR expression in these cells by infecting them with lentiviral constructs that silenced or overexpressed HuR expression (Figure 6). With these constructs, we were able to inhibit HuR protein expression in the cells efficiently, although overexpression of HuR protein in the cells was only modest (Figure 6A-C). At mRNA level, HuR expression was inhibited by 87% and overexpressed by 2-fold (Figure 6D).

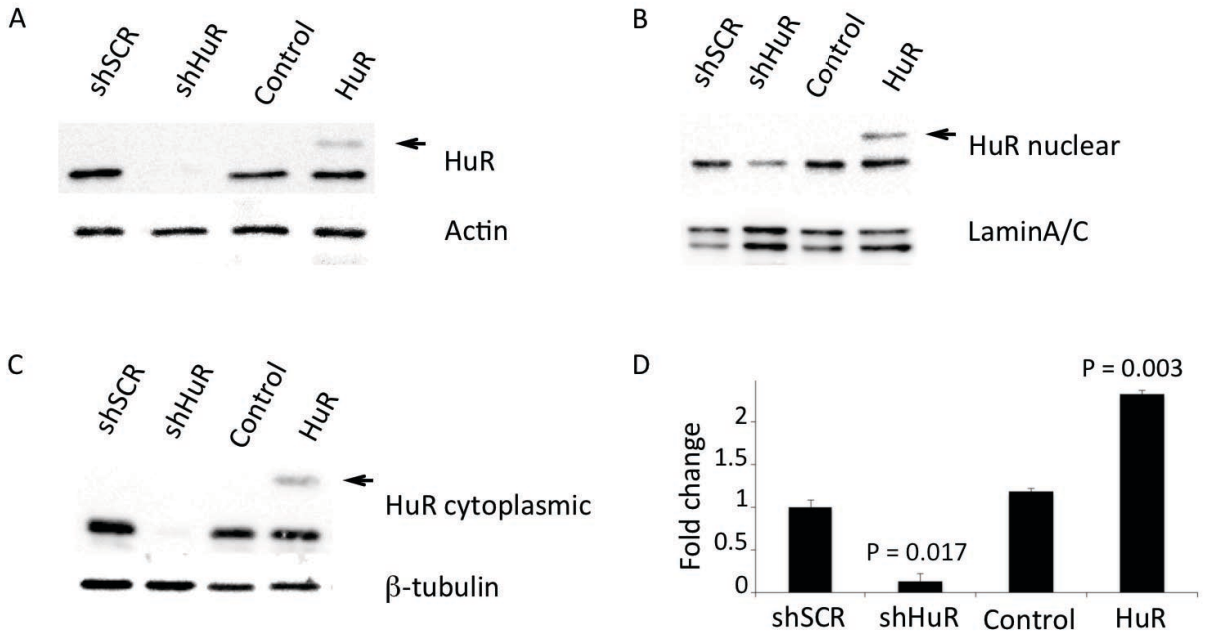


Figure 6. HuR expression in the modified 184B5Me cells. (A) Total protein expression, (B) nuclear HuR protein expression, and (C) cytoplasmic HuR protein expression in the cells. (D) qRT-PCR analysis of HuR levels in the modified cells. shSCR is a control cell line for silenced HuR cells (shHuR), and Control is a control for HuR-overexpressing cells (HuR). An arrow in A-C represents exogenous His-tagged HuR, and the band below this comes from the endogenous HuR protein expression.

To study the malignancy of the cells, we performed an *in vivo* experiment in which HuR-silenced 184B5Me cells (n = 10), and their controls (n = 10) were orthotopically inoculated in Matrigel into NUDE mice mammary fat pads; we monitored tumor formation for 12 weeks (Figure 7A). One control mouse showed slightly atypical cell growth in the mammary gland (Figure 7B) during this monitoring period, while in others, inoculated breast epithelial cells regressed, and connective tissue formation increased (Figure 7C). Based on this, we concluded that these cells do not form tumors *in vivo*, or they form them after a very long lag-period (> 12 weeks). For this reason, we believe that the 184B5Me cell model is representative of DCIS, since these cells are readily transformed but are unable to form invasive tumors in *in vivo* conditions. This is thus a suitable model for study of the mechanisms of HuR in carcinogenesis of the breast.

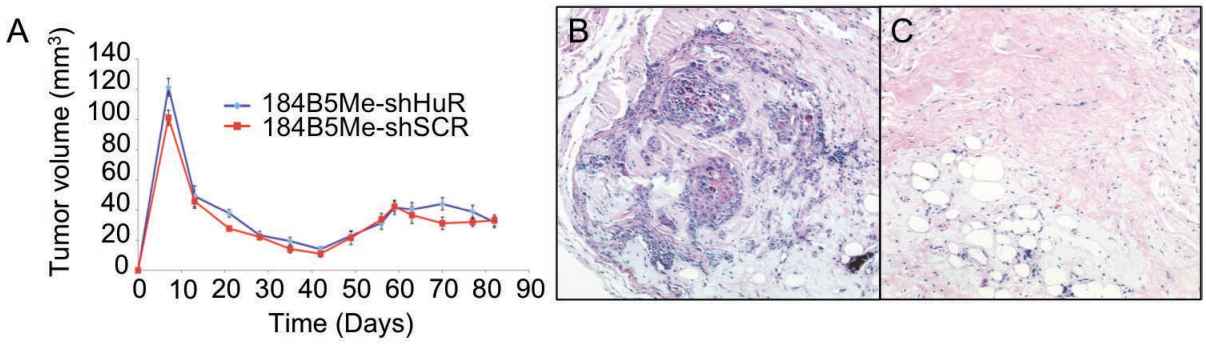


Figure 7. HuR-silenced 184B5Me cells and their control cells growth *in vivo*. (A) HuR-silenced 184B5Me cells and their control cells growth in the mice mammary glands during the 12-week monitoring period. (B) In one case, control cells showed DCIS-like growth in the mouse mammary gland. (C) Orthotopical inoculation of the breast epithelial cells in the mice mammary glands caused regression of the inoculated cells and increased connective tissue growth.

2.1 Mechanisms of HuR to mediate carcinogenesis

Neither overexpression nor silencing of HuR affected cell proliferation in two-dimensional cell culture conditions (Figure 8), suggesting that in this model HuR does not mediate carcinogenic properties by promoting cell growth. Quantitated RT-PCR analysis of COX-2, p21 and p53, genes that mediate cell survival and proliferation and are known targets for HuR regulation (Figure 5), support this hypothesis, since no differences emerged in the expression of these genes (data not shown). Moreover, our clinical DCIS data support this finding, since HuR expression did not associate with the proliferation marker MIB-1. However, HuR regulates expression of genes playing a role in cell survival, and altered HuR expression has been associated with changes in cell-growth properties (Dormoy-Raclet *et al*. 2007, Kang *et al*. 2008). Nevertheless, many of the studies have involved invasive cancer cells, and the role of HuR regulation may differ at the preinvasive stage from that in invasive cells, as also suggested by Mazan-Mamczarz *et al*. (2008a, 2008b) based on their results from breast epithelial and cancer cell microarray analysis.

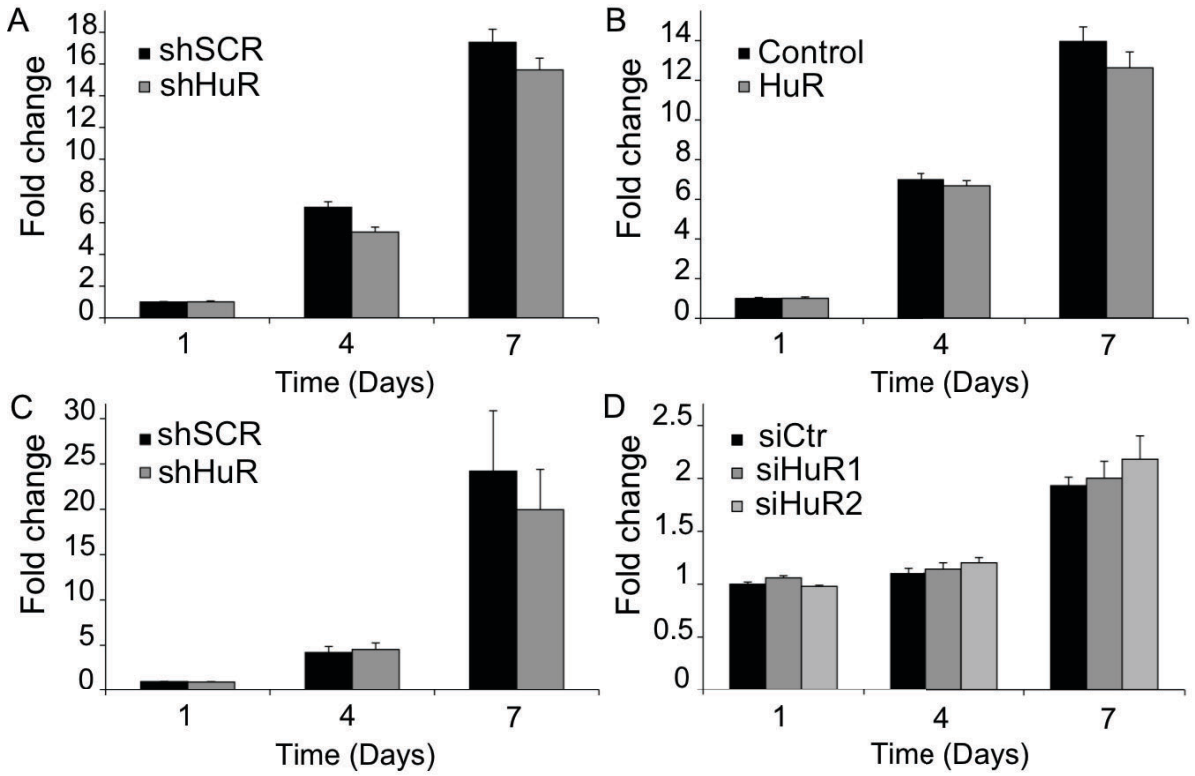


Figure 8. Proliferation of HuR expression-modified 184B5Me cells. Proliferation of HuR-silenced cells and their controls (A) and HuR-overexpressing cells and their controls (B) as measured by CellTiterBlue. (C) Proliferation of HuR-silenced cells and their controls was measured also by cell counting. (D) Transcient HuR siRNA transfections allowed detection of the immediate early effect of HuR silencing on cell proliferation. shSCR is a control cell line for silenced HuR cells (shHuR) and Control is a control for HuR-overexpressing cells (HuR); siCtrl is a control for HuR siRNA transfections, and siHuR1 and siHuR2 present the two different HuR siRNAs.

Interestingly, HuR silencing reduced and overexpression increased cells' ability to grow anchorage-independent (Figure 9A), suggesting that HuR participates in cell transformation and plays an important role in contact-independent growth in breast carcinogenesis. Our result is supported by the finding of Mazan-Mamczarz *et al.* (2008b) showing that HuR depletion in the MCF10A breast epithelial cell line causes a significant reduction in anchorage-independent colony formation. Although when cell proliferation was studied in the cells' actively growing phase the results showed no differences, we decided to investigate the possibility that HuR silencing leads more quickly to cellular senescence, a state in which cell replication has ended, since reduction of HuR expression in fibroblasts has been associated with this phenomenon (Wang *et al*. 2003). However, reduced colony formation after HuR silencing was not a consequence of cellular senescence caused by HuR depletion (Figure 9B and 9D); rather the diminished ability of HuR-silenced cells to grow anchorage-independently was, at least in part, due to increased anoikis, i.e. cell death caused by cell homelessness (Figure 9C). Interestingly, no differences were observable between HuR-overexpressing cells and their controls (Figure 9C), suggesting that normal HuR expression is sufficient to protect

cells from anoikis, whereas overexpression of HuR failed to enhance the protective effect. Although we did not study the mechanisms behind this detachment-induced cell death more carefully, Bcl-2, p53, and Fas receptor-mediated signaling pathways have been shown to mediate the progression of anoikis (Frisch *et al.* 2001, Grossmann 2002), all known target transcripts for HuR regulation (Mazan-Mamczarz *et al.* 2003, Abdelmohsen *et al.* 2007a, Izquierdo 2008, 2010, Ghisolfi *et al.* 2009). These are interesting observations, since when a cancer cell invades or metastasizes to a distant tissue it needs to overcome several challenges like growing in foreign surroundings and evading numerous death signals caused by anoikis and amorphosis (loss of contact with extracellular matrix, ECM) (Psaila *et al.* 2009). Thus this is the first time HuR expression is shown to play a role in anoikis.

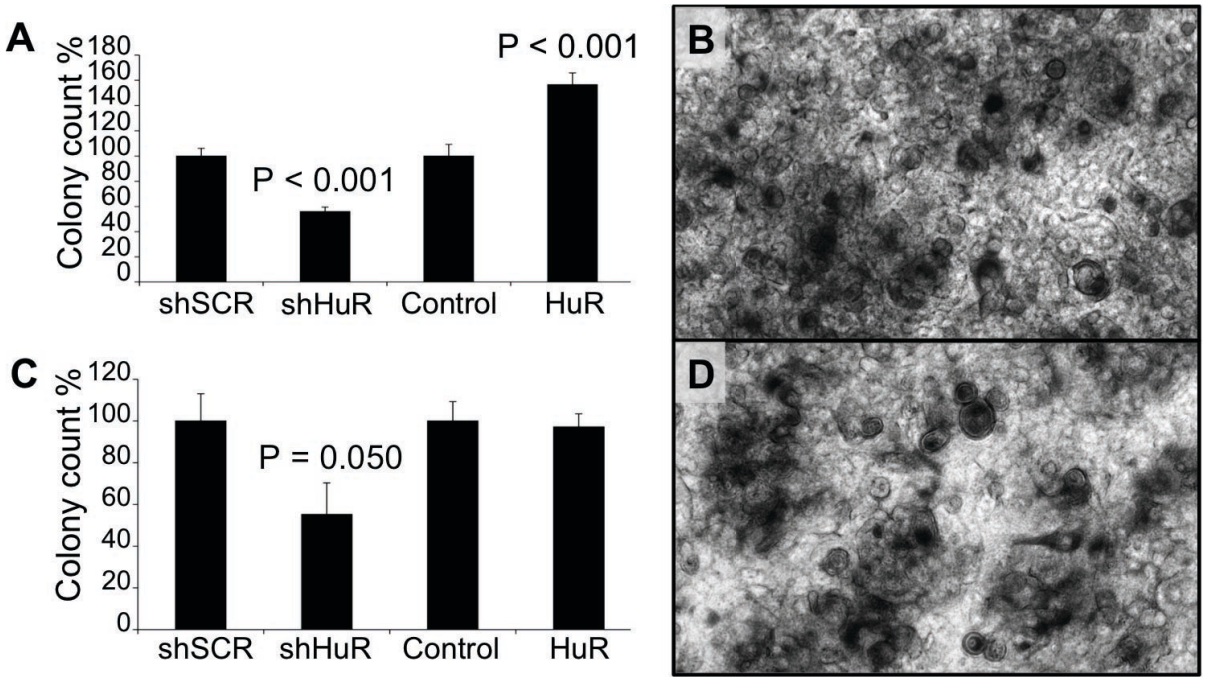


Figure 9. (A) HuR expression-modified 184B5Me cells' ability to grow anchorage-independent. (B) Cellular senescence in HuR-silenced control cells. (C) Programmed cell death caused by anoikis measured in HuR expression-modified 184B5Me cells. (D) Cellular senescence in HuR-silenced 184B5Me cells. In (B) and (D), black represents β-galactosidase positivity. Original magnification in (B) and (D) is 200x.

Since HuR seemed to affect cell-ability to grow without contact with their surroundings, we investigated whether HuR silencing also affects cell motility. Since the microenvironment of the tissue affects cell behavior, we decided to use a myoma organotypic invasion model (Nurmenniemi *et al.* 2009), in which cells are allowed to invade in the uterine leiomyoma tissue in their own culture media without any stimuli. Leiomyoma tissue is composed of collagens, laminins, fibroblast, smooth muscle actin, lymphocytes, macrophages, and endothelial cells forming a more natural stroma-like environment for the epithelial cells. Our results show that HuR-silenced cells' invasion ability was reduced by 25% compared to that

of the control cells. Similarly, in human breast and cervical carcinoma cells, HuR depletion reduces cell migration and invasion ability (Dormoy-Raclet *et al.* 2007, Woo *et al.* 2010). Since HuR regulates EMT by stabilizing Snail mRNA, which leads to an enhancement of cell migration through repression of E-cadherin expression in breast cancer cells (Dong *et al.* 2007), the expression of common EMT-related genes was worth study. However, we found no differences in the expression of E-cadherin, Snail, Slug, Twist, Vimentin, Zeb1, Zeb2 or β-catenin between HuR-silenced cells and their controls (data not shown). Since the traditional HuR targets studied show no difference in their expression and cannot explain the phenomena found, our results suggest a new model for HuR as to how it mediates cell transformation and promotes cell survival, and possibly also disease progression.

2.2 Gene expression arrays and interaction analysis

To better understand the role of HuR in breast carcinogenesis, we studied the effect of HuR silencing on gene expression profiling. Since we were primarily interested in HuR-upregulated genes in breast carcinogenesis, we concentrated on those transcripts, whose expression was inhibited by shHuR. After strict elimination of the genes ($p < 0.01$, genes containing a putative HuR-binding motif, and playing a known role in breast carcinogenesis), we chose ten for further validation. Seven of the validated genes proved not to be direct targets of HuR binding, yet, since HuR silencing inhibits the expression of these genes, they are downstream targets of HuR regulation. However, we identified three genes (ATF2, CTGF, RAB31) to be direct binding targets of HuR based on an HuR-binding assay (immunoprecipitation). Interestingly, these genes have been linked to apoptosis, proliferation, migration, and invasion in breast cancer (Chen *et al.* 2007, Kotzsch *et al.* 2008, Knippen *et al.* 2009). However, although these target genes mediated functions altered in this study, we cannot conclude that the main targets of HuR are any of these three genes, and actually a more likely scenario is that these results are a sum of several factors acting together.

For a comprehensive picture of alterations in the breast epithelial cells caused by HuR silencing, gene network analysis followed using the computational platform Moksiskaan (Laakso *et al.* 2010). Biological processes affected after HuR silencing were related to epithelial cell development, cell adhesion, nucleosome assembly, wounding, response to chemical stimuli, and regulation of biological quality (Table 7). Kyoto Encyclopedia of genes and genomes (KEGG) pathway analysis incorporated genes that were altered after HuR silencing into joint pathways that supported the relationships between the genes. In 20 pathways, significant ($p \leq 0.05$) enrichment was noticeable in genes that showed altered expression after HuR silencing. Enriched KEGG pathways were the TGF-β-signaling pathway, steroid hormone biosynthesis, natural killer cell-mediated cytotoxicity, cytokine-cytokine receptor interaction, apoptosis, metabolism of xenobiotics by cytochrome P450, ECM-receptor interaction, the Wnt-signaling pathway, glycerolipid metabolism, pathways in cancer, dilated cardiomyopathy, gastric acid secretion, amoebiasis, vasopressin-related water reabsorption, the complement and coagulation cascade, axon guidance, the p53 signaling

pathway, the MAPK signaling pathway, glycerophospholipid metabolism, and sphingolipid metabolism. For HuR, p53 and TGF-β are known targets (Nabors *et al*. 2001, Mazan-Mamczarz *et al*. 2003). Similarly, HuR regulates transcripts related to the Wnt signaling pathway (Briata *et al*. 2003, Leandersson *et al*. 2006, Lee *et al*. 2006, Mukherjee *et al*. 2009), and kinases in the MAPK pathway play a role in HuR regulation as well participate in HuR-mediated regulation (Tran *et al*. 2003, Lafarga *et al*. 2009, Abdelmohsen *et al*. 2010a). Mazan-Mamczarz *et al*. (2008a, 2008b) showed that the HuR association changes with a more tumorigenic phenotype: in breast epithelial cells, HuR enhanced expression of genes affecting pathways involved in cellular transformation, and later in breast carcinogenesis, HuR altered its mRNA interaction to genes promoting cancer development. Our data show that HuR regulates several target transcipts in the cells. Together these data suggest a multifactorial role for HuR in the cells as a regulator of several genes in different signaling pathways, and supports the hypothesis of HuR's playing a role in carcinogenesis of the breast.

Table 7. Altered biological processes after HuR silencing in 184B5Me cells.

Description	Ratio	P-value	Down-regulated genes	Up-regulated genes
Ectoderm development	0.069	0.009	CTGF, FOXQ1, FST, KRT34, TGFB2	AHNAK2, GRHL3, IVL, KRT1, KRT13, KRT16, KRT6B, S100A7, SPINK5, SPRR1A, SPRR1B
Epidermis development	0.065	0.009	CTGF, FOXQ1, FST, KRT34, TGFB2	AHNAK2, GRHL3, IVL, KRT1, KRT13, KRT16, S100A7, SPINK5, SPRR1A, SPRR1B
Cell adhesion	0.126	0.023	AMIGO2, CTGF, EPDR1, ITGA4, NRCAM, PCDH20, PPAP2B, PVRL3, S1PR1, SGCE, SPOCK1, SPP1, TGFB2, TNC, VCAN	ARHGDIB, LY6D, CD36, CDH16, COL2A1, DSC1, DSC2, DSG1, FLRT3, GPNMB, PCDHB10, PKP1, SPINK5, WNT4
Nucleosome assembly	0.043	0.023	HIST1H4D, NAP1L3	HIST1H1C, HIST1H2AC, HIST1H2BC, HIST1H2BD, HIST1H2BF, HISTH2BK, HIST1H4H, RTN1
Response to chemical stimuli	0.208	0.023	AGPS, ANGPTL4, CCNA1, CMTM7, CPS, CTGF, CYP1B1, DKK1, DUSP4, DUSP6, EPS8, FKBP1B, GCLM, GNG11, GPAM, GRB14, HDAC4, HSD17B2, LIPG, MTUS1, PLOD2, PRKACB, S1PR1, SERPINA1, SPARC, SPP1, STEAP2, TGFB2	ABCG1, ASS1, BLMH, CD36, CRYAB, CTSO, CYP1A1, CYP4B1, GAL, GUCY1A3, IL1RN, KRT1, KRT13, LCN2, LOX, S100A7, S100A8, S100A9, SULT1B1, WNT4
Response to wounding	0.113	0.023	CFH, CTGF, HDAC4, PPAP2B, RAB27A, S1PR3, SAAL1, SERPINA1, SPP1, TFPI2, TGFB2, TNC, VCAN	ACE2, ASS1, CD36, CYP1A1, GAL, GRHL3, IFNGR1, IL1RN, KRT1, LOX, S100A8, S100A9, SERPINA3

Table 7. Altered biological processes after HuR silencing in 184B5Me cells continues.

Description	Ratio	P-value	Down-regulated genes	Up-regulated genes
Response to organic substances	0.147	0.023	CCNA1, CPS1, CTGF, CYP1B1, DUSP4, DUSP6, EPS8, FKBP1B, GNG11, GPAM, GRB14, HDAC, MTUS1, PRKACB, SERPINA1, SPARC, SPP1, STEAP2, TGFP2	ABCG1, ASS1, CD36, CRYAB, CTSO, CYP1A1, GAL, GUCY1A3, IL1RN, LCN2, LOX, S100A7, S100A8, S100A9, WNT4
Response to herbicides	0.013	0.031		CYP1A1, GUCY1A3, LCN2
Phospholipid homeostasis	0.013	0.038	GPAM, LIPG	ABCG1
Regulation of biological quality	0.216	0.038	ANGPTL4, CDA, CPS1, CTGF, CYP1B1, FKBP1B, FST, GABRA2, GCLM, GPAM, IGFBP6, LIPG, NRCAM, PPAP2B, RAB27A, S1PR1, S1PR3, SERPINA3,1, SHISA9, SLC22A4, SLC30A7, SOAT1, SPP1, TFPI2, TGFB2, TMX3	ABCG1, ACE2, CALB1, CD36, COL2A1, CRYAB, CYP1A1, DSG1, FGFR3, GAL, GUCY1A3, HTRA1, IFNGR1, IGFBP3, IL1RN, KIF26A, KRT1, LCN2, MAFB, NMU, S100A7, SERPINA3, SULT1B1, WNT4
Cell-cell adhesion	0.065	0.040	AMIGO2, CTGF, ITGA4, NRCAM, PCDH20, PPAP2B, PVRL3, TGFB2	CDH16, COL2A1, DSC1, DSC2, DSG1, PCDHB10, WNT4

Ratio is the proportion of the annotated genes in the whole gene set.
Cut-offs used were ≤1/1.5 or ≥1.5 fold, p-value ≤ 0.05.

CONCLUDING REMARKS

The aim of this thesis was to study the prognostic value of HuR in breast cancer and to investigate how HuR facilitates malignant behavior. Understanding the role of HuR in carcinogenesis would optimally provide novel targets to treat breast cancer patients.

Cytoplasmic HuR expression was already elevated in ADH, the earliest non-invasive breast cancer alteration studied, and the expression remained elevated in DCIS as well in invasive cancers. Cytoplasmic HuR expression associated with the more aggressive form of the disease in DCIS, i.e. high grade and microinvasive or axillary node-positive findings. In sporadic and familial non-BRCA1/2 related breast cancer cases, cytoplasmic HuR expression proved an independent marker of reduced survival. Additionally, subgroup analysis of the invasive ductal breast cancer specimens showed that cytoplasmic HuR expression associated with reduced survival in small tumor size and lymph node-negative patients in whom the need for prognostication is great. These data suggest that cytoplasmic HuR expression plays a role in breast carcinogenesis and could be useful in clinics to predict disease prognosis.

HuR silencing led to reduction in anchorage-independent cell growth, to increased programmed cell death, and to decreased invasion of the cells, suggesting that HuR is oncogenic and facilitates carcinogenesis in the breast. Global gene expression analysis showed HuR regulation to be extensive and to affect diverse cancer development-related pathways. HuR-mediated gene regulation means for the cancer cell a rapid and efficient method to alter that cell's microenvironment to become preferable for cancer development. Additionally, we identified two novel target transcripts (CTGF and RAB31) for HuR.

In conclusion, HuR plays a role in breast carcinogenesis by participating in processes important in cell transformation and invasion, and in programmed cell death, suggesting that breast cancer patients could benefit from treatment preventing cytoplasmic HuR expression in their cancer cells. Additionally, cytoplasmic expression of HuR could serve in clinics as a prognostic marker to predict disease progression.

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