Current evidence and future perspectives on HuR and breast cancer development, prognosis and treatment

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Abstract

Hu-antigen R (HuR) is an RNA-binding post-transcriptional regulator that belongs to the Hu/ELAV family. HuR expression levels are modulated by a variety of proteins, microRNAs, chemical compounds or the microenvironment and in turn HuR affects mRNA stability and translation of various genes implicated in breast cancer formation, progression, metastasis and treatment. The aim of the present review is to critically summarise the role of HuR in breast cancer development and its potential as a prognosticator and a therapeutic target. In this aspect, all the existing English literature concerning HuR expression and function in breast cancer cell lines, *in vivo* animal models and clinical studies is critically presented and summarized. HuR modulates many genes implicated in biological processes crucial for breast cancer formation, growth and metastasis, while the link between HuR and these processes has been demonstrated directly *in vitro* and *in vivo*. Additionally, clinical studies reveal that HuR is associated with more aggressive forms of breast cancer and is a putative prognosticator for patients' survival. All the above indicate HuR as a promising drug target for cancer therapy; nevertheless, additional studies are required to fully understand its potential and determine against which types of breast cancer and at which stage of the disease a therapeutic agent targeting HuR would be more effective.

Keywords

Breast cancer; Hu-antigen R; Metastasis; Prognosis; Tamoxifen; Therapeutic target

Introduction

Hu-antigen R (HuR) or ELAV (embryonic lethal, abnormal vision, Drosophila)-like protein 1 (ELAVL1) belongs to the Hu/ELAV family and is a ubiquitously expressed RNA-binding post-transcriptional regulator [1]. Early studies performed using the neuronal-specific HuR orthologue HuB demonstrated that members of the Hu/ELAV family contain three highly conserved RNA binding domains that belong to the RNA recognition motif (RRM) superfamily [2]; RRM-1 and -2 bind to AU-rich elements (ARE), while RRM-3 binds to the poly(A) tail of rapidly degrading mRNAs [3]. Similarly, a U-rich sequence approximately 17-20 nucleotides (nt) long, usually located within the 3' untranslated region (UTR) of the target mRNAs, has been identified as the RNA motif recognised by HuR [4]. HuR binds to this motif and regulates the stability, translation and nucleo-cytoplasmic translocation of target mRNAs. More specifically, HuR binding may stabilize the mRNA, indirectly increasing protein production [5-8], while its direct effect on translation efficiency can be either positive or negative [9, 10]. Moreover, mRNA exon-intron splicing and polyadenylation, processes taking place in the nucleus, can also be modulated by HuR [11-14]. Additionally, HuR can be transported from the nucleus, where is most abundantly localized, to the cytoplasm, along with bound mRNA [15] and this change in subcellular localization appears to be linked to regulating HuR function [16].

The regulatory mechanisms involved in HuR expression and function have not been comprehensively studied. It appears that phosphorylation plays an important role in its subcellular localisation and activity and a number of kinases have been found to phosphorylate HuR, including serine/threonine-protein kinase Chk2 and protein kinase C delta (Table 1). Besides phosphorylation there is also evidence for HuR methylation [17]; however the role of this type of post-translational modification is less well-studied. HuR mRNA and protein levels are altered in response to a number of proteins and microRNAs, such as miR-519 [18], hormones, such as 17β-estradiol [19], cyclic GMP-elevating agents, such as nitric oxide [20], and drugs. Furthermore, HuR protein is degraded *via* the ubiquitin-proteasome system [21] and undergoes caspase-mediated cleavage during apoptosis [22].

Alterations in HuR expression levels or subcellular localization have been associated with important medical conditions, such as pathologic inflammation [23], atherosclerosis [24], tissue ischemia [25] and, most significantly, tumour formation, growth, and metastasis [26-29]. Furthermore, HuR appears to be responsible for the expression regulation of mRNAs encoding proteins involved in transcription, cell signalling, the cell division cycle, apoptosis, inflammation and stress responses [5, 10, 30-33], many of them cancer-relevant and implicated in malignant transformation. Moreover, clinical studies show that increased HuR expression levels and cytoplasmic expression pattern correlate with malignant phenotype and poor patient prognosis in various types of cancer [34].

Breast cancer is the most commonly reported malignancy and the most common cause of cancer-related death amongst women. Mammary tumours are highly complex and heterogeneous and we still lack a global understanding of the molecular mechanisms behind breast cancer origin and progression [35]. Breast cancer cells are classified as either positive or negative for the presence of each of three important receptors: estrogen receptor-alpha (ER), tyrosine kinase-type cell surface receptor HER2 and progesterone receptor. Approximately 70% of breast tumours are ER-positive and depend on estrogen for growth [36]. Therefore, ER-targeted endocrine therapies are effective for the treatment of patients with ER-positive breast tumours and tamoxifen is the most widely used endocrine anti-estrogen treatment. Interestingly, a number of studies implicate HuR in ER and HER2 expression regulation and tamoxifen resistance, suggesting that HuR may play a crucial role in breast cancer development and possibly treatment [37, 38].

In the light of the above considerations, the aim of the current review is to critically summarise the role of HuR in breast cancer as illustrated by *in vitro* experiments, *in vivo* animal models and clinical studies, and to examine its potential as a therapeutic target. Initially, we present an overview of HuR expression and general function in various breast cancer cell lines. Subsequently, we examined individual gene products modulated by HuR either directly as demonstrated by physical interaction between HuR and the target mRNA or in some cases indirectly. Finally, we describe a number of HuR expression regulators including microRNAs and commonly used therapeutic drugs against breast cancer.

Table 1: Regulation of HuR localisation and activity *via* phosphorylation.

HuR modification site	Kinase	Effect on …	Ref.
Ser88	Serine/threonine-protein kinase Chk2	target RNA stability target RNA splicing	[14, 39, 40]
Ser100	Serine/threonine-protein kinase Chk2	target RNA stability target RNA splicing	[14, 39, 40]
Thr118	Serine/threonine-protein kinase Chk2 Mitogen-activated protein kinase 14	HuR localisation target RNA stability target RNA splicing	[14, 39, 41]
Ser158	Protein kinase C alpha	HuR localisation target RNA stability target RNA translation	[42, 43]
Tyr200	Tyrosine-protein kinase JAK3	HuR localisation target RNA stability	[44]
Ser202	Cyclin-dependent kinase 1	HuR localisation	[45]
Ser221	Protein kinase C delta	HuR localisation target RNA stability target RNA translation	[43, 46, 47]
Ser318	Protein kinase C delta	HuR localisation target RNA stability target RNA translation	[46-48]

HuR expression in breast cancer

HuR expression has been studied in a variety of breast-derived cell lines exhibiting differential degrees of malignant potential. Both MCF10A and MCF12A are non-tumorigenic immortalised epithelial cell lines and are considered to be normal breast cells. The MCF7 cell line is epithelial adenocarcinoma, estrogen receptor (ER)-positive. The MDA-MB-231 cell line is also epithelial adenocarcinoma, ER-negative, poorly differentiated and highly tumorigenic and invasive.

HuR expression has been reported in all the above cell lines and HuR mRNA levels in MDA-MB-231 cells are 2.5-fold higher than in MCF7 cells and 5-fold higher than in MCF10A and MCF12A cells, as shown by RT-qPCR [49]. HuR mRNA was also found to be more stable in MDA-MB-231 cells as compared to MCF10A cells, with its half-life increasing from 1 h in the latter to 4 h in the former [49].

Although HuR is mainly localised to the nucleus [50, 51], immunochemical studies revealed increased cytoplasmic HuR expression in MDA-MB-231 in comparison with MCF7 cells [52], as well as in MCF7 in comparison with MCF10A cells [53]. Interestingly, Hostetter *et al.* report that total HuR protein levels are higher in MCF7 than in MDA-MB-231 cells [37], while Calaluce *et al.* report similar HuR protein levels in MCF7 and MDA-MB-231 cells [52].

HuR expression has been also noted in non-tumorigenic immortalised epithelial HB2 [50] and HMT-3522-T4-2 [54] cells, epithelial ductal carcinoma T47D [50, 54-56] and ZR-75-1 cells [57], epithelial carcinoma BT-20 [51] and Hs578T cells [58, 59] and epithelial adenocarcinoma SK-BR-3 cells [38, 51, 54, 60, 61].

A number of clinical studies revealed that HuR expression levels were elevated in atypical ductal hyperplasia (ADH), ductal carcinoma *in situ* (DCIS) and ductal invasive carcinoma (DIC) when compared to healthy tissue samples [62-64]. Interestingly, cytoplasmic HuR immunoreactivity was present in less than half of DIC [65-67], DCIS and ADH samples [64], as well as in invasive breast cancer patients that underwent paclitaxel and anthracycline-based neoadjuvant chemotherapy (NACT) [68]. The cytoplasmic localisation of HuR in histological and cytological samples of invasive breast carcinoma is evident in Figure 1.

Apart from the apparent association between increased HuR mRNA levels and cytoplasmic HuR expression and a more malignant phenotype, HuR has been reported to regulate different biological processes in different cell lines.

HuR knockdown in MCF10A cells revealed that HuR plays a crucial role in cell proliferation as demonstrated by a colony formation assay, reducing the number of colonies by up to 28%, and promoting premature senescence [69]. In another study, siRNA-mediated HuR silencing decreased

anchorage-independent growth of malignant T-cell-amplified sequence 1 (MCT1)-transformed MCF10A cells [70]. Additionally, HuR regulates cell polarity and is responsible for the formation of the acinar structures of MCF10A cells in 3-D Matrigel culture [69]. Similarly, HuR knockdown was found to significantly decrease growth of MCF7 but not MDA-MB-231 cells [57], while a second study confirmed this observation reporting a 35% reduction in MCF7 cell number after siRNA-mediated HuR silencing [71]. However, in another study, HuR over expression in MDA-MB-231 cells was shown to enhance growth rate by altering cell cycle kinetics and increasing the number of cells in G_1 (67 vs 57%), while decreasing the number of cells in the G_2/M phase (18 vs 27%) [72]. Gubin *et al.* also used an orthotopic xenograft mouse model and demonstrated that HuR over expression results in significantly reduced tumour growth and mass by 90%, as confirmed by MRI scans, gross photographs and microscopy. Both tumours were classified as moderately to poorly differentiated carcinoma, but HuR-over expressing tumours appeared to be gelatine-like capsules with a smooth, homogeneous and glistening surface, whereas the control tumours were a solid round mass and had a heterogeneous, yellow-white surface with a necrotic centre [72].

In addition, HuR knockdown reduces invasiveness of MDA-MB-231 but not MCF7 cells, as shown by a Matrigel invasion assay [57]. In confirmation, siRNA-mediated HuR silencing was shown to decrease the invasion ability of MDA-MB-231 cells 1.4-fold, while HuR over expression increases their invasion ability 2.0-fold. Similar results were noted in BT-20 cells [73]. More recently, siRNA-mediated HuR silencing was also shown to reduce invasiveness of MDA-MB-231 cells by 72% [49].

Moreover, siRNA-mediated HuR silencing decreases the motility of BT-20 cells 1.6-fold while HuR over expression increases their motility 2.4-fold [73]. In contrast, HuR knockdown does not affect cell adhesion or migration in both MCF7 or MDA-MB-231 cells [57] and HuR over expression does not appear to affect apoptosis in MDA-MB-231 cells or in an orthotopic xenograft mouse model [72].

HuR target genes

HuR exerts its effects on cell proliferation, invasion ability and motility of mammary cell lines by regulating the expression of target genes (Table 2).

Two major types of studies have been conducted in order to elucidate the global profile of HuR target genes, using immunoprecipitation of ribonucleoprotein complexes and microarray analysis (RIP-Chip assay), as initially described for HuB, another member of the Hu/ELAV family [74].

The first study assessed the HuR targets in comparison with the targets of the heterogeneous nuclear ribonucleoprotein D0 (HNRNPD/AUF1), in malignant T-cell-amplified sequence 1 (MCT1)-transformed compared to non-transformed immortalized MCF10A cells. In total, 1,676 and 2,072 mRNAs exhibited significantly altered binding to HuR or HNRNPD/AUF1, respectively, and 712 of

these mRNAs exhibited differential binding to both proteins. Functionally, these genes are mostly associated with cell cycle regulation, signal transduction, DNA damage response, translation regulation and angiogenesis, as demonstrated by Gene Ontology (GO) analysis. Using the KEGG pathway database, nine pathways were found significantly enriched in these genes: cell cycle, cell communication, p53 signalling pathway, ribosome, oxidative phosphorylation, purine metabolism, focal adhesion, ubiquitin-mediated proteolysis and regulation of actin cytoskeleton. Similarly, in the BioCarta pathway database, seven pathways were significantly enriched: caspase cascade in apoptosis, cyclins and cell cycle regulation, Erk1/Erk2 MAPK signalling, Erk and phosphatidylinositol 3-kinase are necessary for collagen binding in corneal epithelia, role of Ran in mitotic spindle regulation, phosphoinositides and their downstream targets and HIV type I Nef (negative effector of Fas and tumour necrosis factor - TNF) [70]. In parallel, Mazan-Mamczarz *et al*. applied a similar methodology to MCF7 cells. Overall, *ca.* 9,000 mRNAs were bound by HuR and 595 of them exhibited significantly altered binding to HuR in MCT1-transformed compared to non-transformed MCF7 cells. Functional analysis revealed that these genes are implicated in cell cycle arrest, apoptosis and angiogenesis, together with pathways important for cell survival and proliferation [75]. Overall, these results support the notion that HuR plays an important role as a regulator of key gene expression during malignant transformation.

In the second type of study HuR targets were compared in MDA-MB-231 and MCF7 cells. 395 and 64 annotated genes were respectively identified as HuR targets, together with 182 genes in both cell lines. GO analysis of the genes differentially bound by HuR in MDA-MB-231 and MCF7 cells revealed that they are implicated in epithelial cell differentiation, hormone metabolism, regulation of biological processes, blood vessel morphogenesis, anatomical structure formation, vasculature development, nucleic acid metabolism, macromolecule biosynthesis, regulation of metabolic processes, transcriptional regulation, regulation or cellular processes and signal transduction [52].

Besides these high throughput studies, the physical interaction of HuR with individual genes was demonstrated by immunoprecipitation. Additionally, immunoblotting, northern hybridisation, qPCR amplification or luciferase reporter genes were all regularly used in transcription and cell signalling assays in order elucidate the regulatory mechanism of gene expression.

Table 2: Effect of HuR protein on mRNA stability and/or translation of genes in breast cancer cell lines

Protein	Effect	Cell line	Ref.
Transcription			
Estrogen receptor (ESR1)	mRNA stabilization↑	MCF7	[37, 76]
Trans-acting T-cell-specific transcription factor GATA-3 (GATA3)	mRNA stabilization↑	MCF7 BT474	[71]
Forkhead box protein O1 (FOXO1)	mRNA stabilization↑	MDA-MB -231	[77]
Homeobox protein Hox-A5 (HOXA5)	mRNA stabilization↑	MCF7	[78]
Signal transducer and activator of transcription 3 (STAT3)	Expression[a]↑	MCF10A[b]	[70]
Activator protein 1 (AP1)	Expression[a]↑	MCF10A[b]	[70]
Proto-oncogenes c-fos (FOS)	Expression[a]↑	MCF7	[79]
Myc proto-oncogene protein (MYC)	Expression[a]↑	MCF7	[79]
Cell signalling			
Tyrosine protein kinase Yes (YES1)	Expression[a] (?)	MDA-MB -231	[80]
Protein Wnt-5a (WNT5A)	Translation↓	HB2 MCF7	[50]
Insulin growth factor 1 receptor (IGF1R)	Translation↓	T47D MCF10A	[55]
Receptor tyrosine-protein kinase erbB-2 (ERBB2)	mRNA stabilization↑	SK-BR-3	[38]
Calmodulin (CALM2)	mRNA stabilization↑	MCF7	[52]
Suppressor of cytokine signalling 3 (SOCS3)	Expression[a]↑	MCF7	[79]
C-X-C chemokine receptor type 4 (CXCR4)	mRNA stabilization↑	MDA-MB-231	[81]
Cell cycle			
Cyclin-dependent kinase inhibitor 1 (CDKN1A)	mRNA stabilization↑	MDA-MB-468	[82]
Breast cancer type 1 susceptibility protein (BRCA1)	Translation↓	MCF7, T47D	[56]
G_1/S-specific cyclin E1 (CCNE1)	mRNA stabilization↑	MCF7 MCF10A	[53]
Cellular tumour antigen p53 (TP53)	Expression[a]↑	MCF10A MCF7 SK-BR-3	[60, 69]
Protein phosphatase 1D (PPM1D/WIP1)	Expression[a]↑	MCF7 SK-BR-3	[60]
Tumour protein 63 - delta Np63 (TP63)	Translation↓	MCF10A	[69]
Cyclin-dependent kinase 1 (CDK1)	Expression↑	MCF10A[b]	[70]
Cyclin-dependent kinase 7 (CDK7)	Expression↑	MCF10A[b]	[70]
DNA repair protein RAD51 homolog 1 (RAD51)	Expression↑	MCF10A[b]	[70]
Inflammation			
Interleukin-8 (CXCL8)	mRNA stabilization↑	Hs578T	[58]
Macrophage colony-stimulating factor 1 receptor (CSF1R)	mRNA stabilization↑	BT-20 SK-BR-3	[51]
Cyclooxygenase-2 (COX2)	mRNA stabilization↑	MDA-MB-231	[83]
Cell adhesion and angiogenesis			
CD9 antigen (CD9)	mRNA stabilization↑ mRNA stabilization↓	MCF7 MDA-MB-231	[52]
Thrombospondin-1 (THBS1)	mRNA stabilization↑	MCF7[b] MDA-MB -231	[72, 75]
Vascular endothelial growth factor A (VEGFA)	Expression[a]↓ Expression[a]↑	MDA-MB -231 MCF7	[59, 72]
Platelet-derived growth factor-C (PDGF-C)	mRNA stabilization↑	MDA-MB-231	[84]
Matrix metalloproteinase-9 (MMP9)	mRNA stabilization↑	MDA-MB -231	[57]
Apoptosis			
Tumour necrosis factor ligand superfamily member 12 (TNFSF12)	Expression[a]↑	MCF10A[b]	[70]
Apoptosis regulator BAX (BAX)	Expression[a]↑	MCF10A[b]	[70]
Caspase-2 (CASP2)	Expression[a]↑	MCF10A[b]	[70]
Others			
Eukaryotic translation initiation factor 4E-binding protein 2 (eIF4EBP2)	Expression[a]↑	MCF10A[b]	[70]
Ras-related protein Rab-2A (RAB2A)	Expression[a]↑	MCF10A[b]	[70]

[a]The term 'expression' signifies that the exact mechanism of regulation has not been elucidated

[b]MCT-1-transformed cell line

Transcription

Estrogen receptor-alpha (ER) is the major mediator of the mitogenic effects of estrogen in the mammary gland [85]. HuR binds to the 3'-UTR of ER mRNA in MCF7 cells [37] and siRNA-mediated HuR silencing resulted to a down-regulation of ER mRNA levels and half-life [76]. Notably, HuR phosphorylation increases its binding to the 3'-UTR of the ER mRNA; more specifically, phosphorylation at the S88, S100 and T118 sites is necessary for HuR binding to the ER 3'-UTR, as demonstrated by the use of HuR phosphorylation mutants [37].

Trans-acting T-cell-specific transcription factor GATA-3 is implicated in cell differentiation, while its expression has been correlated with ER expression in breast cancer [86]. HuR binds to the 3'-UTR of GATA-3 mRNA in MCF7 cells, while siRNA-mediated HuR silencing decreased GATA-3 mRNA and protein levels in MCF7 and BT474 cells, respectively. In addition, the half-life of GATA-3 mRNA was significantly reduced. Interestingly, siRNA-mediated silencing of either HuR or GATA-3 inhibited cell proliferation by 35% and 44%, respectively [71].

Forkhead box protein O1 (FOXO1) is a transcription factor implicated in response to oxidative stress and HuR binds to the 3'-UTR of FOXO1 mRNA in MDA-MB-231 cells stabilising it and enhancing its half-life. SiRNA-mediated HuR silencing and HuR over expression revealed that HuR significantly increases in FOXO1 mRNA and protein levels [77].

The homeobox protein Hox-A5 has been shown to negatively regulate angiogenesis in breast cancer [87] and HuR binds to the 3'-UTR of the Hox-A5 mRNA in MCF7 cells. Retinoic acid treatment (100 μM) enhances the HuR-Hox-A5 mRNA interaction and subsequently increases Hox-A5 mRNA and protein levels. Induction of Hox-A5 following RA treatment is co-regulated by HuR and miR-130a and HuR-mediated Hox-A5 regulation plays an important role in RA-induced cell death [78].

HuR also binds to the signal transducer and activator of transcription 3 (STAT3) and activator protein 1 (AP1) mRNAs in MCF10A cells [70] and to proto-oncogenes c-fos and c-myc mRNA in MCF7 cells [68].

Cell signalling

The non-receptor tyrosine-protein kinase Yes belongs to the SRC subfamily and has been associated with invasion and metastasis of breast cancer cells. HuR binds to the proximal 3'-UTR (nt 1840-3174) of the Yes mRNA in MB-MDA-231 cells, and appears to play a role in Yes expression regulation [80].

Protein Wnt-5a is a ligand for members of the frizzled family of seven transmembrane receptors and may either activate or inhibit canonical Wnt signalling, depending on receptor context. In breast cancer, Wnt-5a plays a role in cell migration and invasiveness and low Wnt-5a expression levels are correlated with poor prognosis in breast cancer patients [88]. HuR binds to the AU-rich sequences in

the 3’-UTR of Wnt-5a mRNA in HB2 cells. Interestingly, HuR binding does not affect Wnt-5a mRNA stability but suppresses Wnt-5a translation. The phenomenon was also studied under hypoxic conditions (1% O_2, 24 h), which increased HuR protein levels. As expected Wnt-5a mRNA levels remained stable during hypoxia. However Wnt-5a protein levels were significantly reduced, and similar results were obtained in MCF7 cells [50].

Insulin-like growth factor 1 receptor (IGF1R) is a receptor tyrosine kinase which mediates actions of insulin-like growth factor 1 (IGF1), controlling cell proliferation. HuR binds to the IRES located in the 5’-UTR of the IGF-1R mRNA in T47D and MCF10A cells. SiRNA-mediated HuR silencing and HuR over expression revealed that HuR represses IGF1R IRES activity. Interestingly, HuR competes with the heterogeneous nuclear ribonucleoprotein C (hnRNP C) for binding to the IGF1R 5’-UTR and the two proteins exert opposite effects on IGF-1R IRES activity. Amino acid deprivation (16 h) of T47D cells down-regulates HuR protein levels but increases HuR binding to IGF-1R IRES and reduces IRES activity, while induced G_2/M cell cycle arrest (nocodazole, 100 ng/ml, 24 h) up-regulates HuR protein levels but reduces HuR binding and increases IRES activity [55].

Receptor tyrosine-protein kinase erbB-2 (ERBB2), alternatively known as tyrosine kinase-type cell surface receptor HER2 or proto-oncogene Neu, is a protein tyrosine kinase. HuR binds to the U-rich sequence (nt 465-505) in the 3’-UTR of the erbB2 mRNA in SK-BR-3 cells. SiRNA-mediated HuR silencing results in a decrease of erbB2 mRNA and protein levels [38].

Calmodulin is a regulatory protein that has been shown to interact with ER, probably exerting an inhibitory effect [89]. HuR binds to the 3’-UTR of the calmodulin mRNA. SiRNA-mediated HuR silencing and HuR over expression revealed that HuR increases CALM2 mRNA and protein levels in MCF7 cells [52].

Suppressor of cytokine signalling 3 (SOCS3) is involved in negative regulation of cytokines and HuR binds to the (SOCS3) mRNA in MCF7 cells [79].

C-X-C chemokine receptor type 4 (CXCR-4) is a G-protein coupled chemokine receptor over-expressed in breast cancer and is involved in metastasis, invasion and migration of breast cancer cells. HuR binds to the 3’-UTR of CXCR-4 mRNA in MDA-MB-231 cells. SiRNA-mediated HuR silencing resulted in a 50% reduction of CXCR-4 mRNA and protein levels. Both HuR and CXCR-4 expression levels were low in normal tissues and higher in invasive ductal and lobular breast carcinoma ones. Similar results were noted in MCF10A, MCF12A, MCF7 and MDA-MB-231 cells, with the MDA-MB-231 cells expressing 2-fold higher CXCR-4 mRNA levels in comparison with MCF7 cells and more than a 40-fold and a 130-fold higher CXCR-4 mRNA levels as compared to the normal MCF10A and MCF12A cells, respectively. CXCR4 protein levels are also higher in MDA-MB-231 cells as compared to MCF10A. Moreover, CXCR-4 mRNA was more stable in MDA-MB-231 cells as compared to

MCF10A cells. SiRNA-mediated silencing of both CXCR-4 and HuR has significant inhibitory effects on invasion and migration of MDA-MB-231 cells [81].

Tribbles-homolog 3 (TRB3) is a kinase-like protein implicated in stress response. SiRNA-mediated silencing of HuR in MCF7 cells under anoxic conditions (48 h) reduced TRB3 mRNA by 2.4-fold and its half-life by 51% with a concomitant decrease in TRB3 protein levels [90].

Cell cycle

Cyclin-dependent kinase inhibitor 1 (p21) plays an important role in cell cycle progression and acts as an inhibitor of cellular proliferation in response to DNA damage. HuR interacts with the 3'-UTR of the p21 mRNA, in MB-MDA-468 cells. More specifically, HuR binds to the AU-rich WAF1-HuD sequence (nt 657-698) within the WAF1-1/6 region (nt 571-829) of the p21 mRNA. HuR binding was reported to increase after exposure of the cells to short wavelength ultraviolet light (254 nm, 20 J/m^2, 6 h), a mediator of p21 mRNA stability [91] and, under the same conditions, an approximately 70% up-regulation of p21 mRNA stability [82]. In addition, HuR knockdown decreases p21 protein levels in MCF10A cells [69]. This constitutes a post-transcriptional mechanism of regulation of p21 expression levels, in contrast to its transcriptional regulation by p53.

Breast cancer type 1 susceptibility protein (BRCA1) plays a central role in DNA repair by facilitating cellular responses to DNA damage. Notably, BRCA1 gene mutations comprise the most important genetic susceptibility factor for breast cancer. HuR binds to the 3'-UTR (nt 281-315) of the BRCA1 mRNA in MCF7 and T47D cells. Further experiments in HeLa cells indicate that HuR negatively regulates BRCA1 protein levels, with no effect on mRNA levels or stability [56].

G1/S-specific cyclin E1 plays a crucial role in the regulation of cell cycle progression and cell proliferation. Full-length cyclin E1 together with its functional, hyperactive, low molecular weight isoforms are over expressed in breast cancer and HuR binds to the 3'-UTR of cyclin E1 mRNA in both MCF7 and MCF10A cells. SiRNA-mediated HuR silencing in MCF7 cells significantly decreased the half-life of cyclin E1 mRNA and concomitantly full-length cyclin E1 protein levels were reduced by 22% and the low molecular weight isoforms by 80%. As a result, G_1/S cell cycle arrest was noted using flow cytometry, and cell cycle progression and cell proliferation were subsequently restored by over expression of the low molecular weight cyclin E1 isoforms. Similarly, HuR over expression in MCF10A cells increased both cyclin E1 mRNA half-life and doubled protein levels [53]. Cold-inducible RNA-binding protein (CIRBP), which is negatively regulated by HuR while positively affecting HuR protein levels, also increases HuR binding to cyclin E1 mRNA, enhancing its stability [92].

Additionally, a recent study demonstrated that miR-16 blocks HuR-mediated up-regulation of cyclin E1 in MCF7 cells [93].

The cellular tumour antigen p53 acts as a tumour suppressor by inducing growth arrest or apoptosis. HuR binds to p53 mRNA in MCF7 and SK-BR-3 cells enhancing its expression [60]. In addition, HuR knockdown decreases p53 protein levels in MCF10A cells [69]. Interestingly, cytoplasmic HuR expression pattern has been significantly associated with positive p53 immunostaining in familial non-BRCA1/2 patients [94].

Protein phosphatase 1D (PPM1D/WIP1) is implicated in p53-dependent checkpoint mediated cell cycle arrest and HuR binds to PPM1D/WIP1 mRNA in MCF7 and SK-BR-3 cells enhancing its expression [60].

Isoforms of tumour protein 63, designated as delta Np63, are known to suppress cell proliferation. HuR binds to U-rich elements (nt 4010-4220 and nt 4640-4868) in the 3'-UTR of the delta Np63 mRNA in MCF10A cells. HuR knockdown has little effect of delta Np63 mRNA levels but increases protein levels of delta Np63p and its target, growth arrest and DNA damage-inducible protein GADD45 alpha. Delta Np63-knockdown revealed that delta Np63 partly mediates HuR-knockdown-induced growth suppression and premature senescence in MCF10A cells [69].

Moreover, HuR modulates the expression of other gene products implicated in cell cycle regulation and DNA damage response. More specifically, HuR binds the cyclin-dependent kinase 1 (CDK1), cyclin-dependent kinase 7 (CDK7) and DNA repair protein RAD51 homolog 1 (RAD51) mRNAs in MCF10A cells. SiRNA-mediated HuR silencing reduced protein levels of all the above genes [70]. Finally, HuR knockdown correlates with down-regulated G_1/S-specific cyclin-D1 mRNA and protein levels in MCF7 but not MDA-MB-231 cells [57].

Inflammation

Interleukin (IL)-8 is a chemokine that promotes malignant phenotype and metastasis in breast cancer, while produced by tumour cells as a response to other inflammatory cytokines in the tumour microenvironment [95]. In addition, IL-8 is a strong inducer of angiogenesis and it mediates endothelial cell chemotaxis and proliferation *in vitro* and angiogenic activity *in vivo* [96]. HuR binds to the proximal 3'-UTR of IL8 mRNA in in IL1-beta-stimulated Hs578T cells. Stimulation with IL1-beta (5 ng/ml, 6-24 h) also resulted in a time-dependent induction of IL-8 mRNA and protein levels and stabilization of IL-8 mRNA. Taken together, these observations suggest that HuR contributes to IL-8 mRNA stabilization [58].

Macrophage colony-stimulating factor 1 receptor (CSF-1-R), alternatively known as proto-oncogene c-fms, is associated with cell proliferation, metastasis and poor survival [97]. A statistically significant association between high nuclear and cytoplasmic HuR and high cytoplasmic CSF-1-R expression levels has been observed. HuR binds to a 69-nt element which contains five 'CUU' motifs within the

3’-UTR of CSF-1-R mRNA in BT-20 and SK-BR-3 cells. Site-directed mutagenesis confirmed the necessity of these motifs for binding [51]. Interestingly, vigilin, a regulator of lipid metabolism, also binds to the same 69 nt element competing with HuR and exerting negative effects on CSF-1-R mRNA and protein levels [73]. SiRNA-mediated HuR silencing and HuR over expression revealed that HuR increases CSF-1-R RNA and protein levels in both BT-20 and SK-BR-3 cells, respectively. In addition, glucocorticoid stimulation of CSF-1-R expression is largely dependent on the presence of HuR [51].

Cyclo oxygenase (COX)-2, also known as prostaglandin G/H synthase 2 (PTGS2) is responsible for the production of inflammatory prostaglandins. HuR binds to COX-2 mRNA in MDA-MB-231 cells. As a result, COX-2 mRNA is stabilised [83]. Interestingly, increased HuR protein levels were significantly associated with increased COX-2 expression in DIC patients [66].

Cell Adhesion and Angiogenesis

CD9 antigen is implicated in cell adhesion and cell motility, together with tumour metastasis. HuR binds to the 3’-UTR of the CD9 mRNA and differentially regulates its mRNA and protein levels in MDA-MB-231 and MCF7 cells. More specifically, siRNA-mediated HuR silencing and HuR over expression revealed that HuR decreases CD9 mRNA and protein levels in MDA-MB-231 cells, while increasing CD9 mRNA and protein levels in MCF7 cells [52].

The angiogenesis inhibitor thrombospondin-1 (THBS1) is a glycoprotein functioning as a tumour suppressor [98]. THBS1 plasma levels have been positively correlated with breast cancer progression [99]. HuR binds to the terminal the 3’-UTR of THBS1 mRNA in MCF10A and MDA-MB-231 cells [72, 75]. SiRNA-mediated HuR silencing and HuR over expression revealed that HuR modulates THBS1 protein levels. More specifically, a 2-fold decrease or increase of THBS1 protein levels was noted in HuR-silenced and HuR-over expressing MCF7 cells, respectively. Interestingly, the association between HuR and THBS1 mRNA was reduced by 65-85% in MCT1-transformed MCF7 cells [75]. HuR-over expressing tumours in an orthotopic xenograft mouse model exhibited increased THBS1 mRNA (5.44-fold) and protein (76%) levels [72].

Vascular endothelial growth factor A (VEGF-A) is a growth factor involved in angiogenesis. HuR was shown to bind to VEGF-A mRNA in MDA-MB-231 cells. Surprisingly, HuR-over expressing tumours in orthotopic mice exhibited decreased VEGF-A mRNA (2.6-fold) and protein (23%) levels with no alteration in stability [72].

Platelet-derived growth factor (PDGF)-C plays an important role in cell proliferation and migration, and its expression has been associated with poor prognosis in breast cancer patients. HuR and PDGF-C expression levels have been correlated in mammary cell lines and breast cancer patients and direct

binding of HuR to the 3'-UTR of the PDGF-C mRNA has been demonstrated. SiRNA-mediated HuR silencing confirmed the stabilization of PDGF-C mRNA by HuR in MDA-MB-231 cells. HuR-mediated up-regulation of PDGF-C appears to be involved in the responses against ultraviolet irradiation (30 J/m^2) and oxidative stress (H_2O_2, 800 μM, 24 h) in MCF7 cells [84].

Matrix metalloproteinase (MMP)-9 plays an essential role in local proteolysis of extracellular matrix and cell migration. MMP-9 is over-expressed in breast cancer and has been associated with metastasis [100]. HuR knockdown correlates with reduced MMP-9 mRNA and protein levels in MDA-MB-231 but not in MCF7 cells [57].

Hypoxia-induced factor 1 alpha (HIF-1-alpha) is the main transcriptional regulator in response to hypoxic conditions, facilitating metabolic adaptation to hypoxia and playing a crucial role in tumour angiogenesis, with increased HIF-1-alpha expression levels associated with poor patients' survival [101]. SiRNA-mediated silencing of HuR reduced HIF-1-alpha protein levels in MCF7 and Hs578T cells [59].

Apoptosis

HuR positively regulates the expression of gene products promoting programmed cell death. Analytically, HuR binds the tumour necrosis factor ligand superfamily member 12 (TNFSF12), apoptosis regulator BAX, caspase-2 (CASP2) mRNAs in MCF10A cells. SiRNA-mediated HuR silencing reduced protein levels of these genes [70].

Others

Finally, HuR is implicated in the expression regulation of gene products involved in translation, such as the eukaryotic translation initiation factor 4E-binding protein 2 (eIF4EBP2) and the cold-inducible mRNA-binding protein (CIRBP), and in protein transport from the endoplasmic reticulum to the Golgi complex, namely the Ras-related protein Rab-2A (RAB2A). More specifically, HuR binds the eIF4EBP2 and RAB2A mRNAs in MCF10A cells and siRNA-mediated HuR silencing reduced protein levels of these genes [70]. SiRNA-mediated HuR silencing increases both CIRBP mRNA and protein levels by 2-fold in MCF7 cells [92].

HuR modulators

A number of environmental conditions, proteins, microRNAs and drugs have been shown to directly or indirectly modulate HuR expression and activity in mammary cell lines (Table 3).

Ultraviolet irradiation up-regulates HuR protein levels in MCF7 cells in a dose-dependent manner [84]. Furthermore, anoxia (< 0.01% O_2) increases cytoplasmic HuR expression levels in MCF7 cells.

More specifically, a significant translocation of the nuclear HuR to the cytoplasm after 12 h of anoxic incubation and translocation of majority of HuR to the cytoplasm after 24 h was noted [90]. In addition, oxidative stress (H_2O_2, 800 μM, 24 h) and induced G_2/M cell cycle arrest (nocodazole, 100 ng/ml, 24 h) up-regulate HuR protein levels, while amino acid deprivation (16 h) down-regulates HuR protein levels [55, 84].

Table 3: Effect of HuR modulators on HuR expression and function in breast cancer cell lines

Regulators	Effect on HuR	Cell line	Ref.
Environmental conditions			
G_2/M cell cycle arrest	HuR protein levels↑	T47D	[55]
amino acid deprivation	HuR protein levels↓	T47D	[55]
anoxia (< 0.01% O_2)	HuR shuttling↑	MCF7	[90]
ultraviolet irradiation	HuR protein levels↑	MCF7	[84]
oxidative stress	HuR protein levels↑	MCF7	[84]
Proteins			
Breast cancer type 1 susceptibility protein (BRCA1)-IRIS	HuR transcription↑	MCF7 SK-BR-3	[60]
Mitogen-activated protein kinase 8 (MAPK8)	HuR shuttling↑	MCF7 MDA-MB-231 BT474	[37, 102]
Cold-inducible RNA-binding protein (CIRBP)	HuR protein levels↑	MCF7	[92]
Heat-shock factor protein 1 (HSF1)	HuR transcription↑	MCF7 Hs578T	[59]
Protein kinase C (PKC) delta	HuR shuttling↑	MCF7	[61, 79]
Tristetraproline (TTP)	HuR mRNA levels↓	MCF10A MCF12A MCF7 MDA-MB-231	[49]
Epidermal growth factor receptor (EGFR)	HuR binding↑	MDA-MB-231	[83]
microRNAs			
miRNA-125	HuR translation↓	MCF10A MCF7 T47D	[54]
miRNA-16	HuR translation↓	MDA-MB-231	[63]
miRNA-29a	HuR mRNA levels↑	MCF10A MDA-MB-231	[49]
miRNA-7	HuR binding↓	MDA-MB-231	[83]
Drugs			
Trichostatin A (TSA, 100 ng/ml)	HuR shuttling↓	MCF7 MDA-MB-231	[37, 76, 102]
5-aza-2'-deoxycytidine (AZA, 2.5 μM)	HuR shuttling↓	MCF7 MDA-MB-231	[37, 76, 102]
Tamoxifen (2.5 μM)	HuR shuttling↑	MCF7 MDA-MB-231 BT474	[37, 102]
Doxorubicin (10 μM)	HuR shuttling↑	MCF7	[61, 79]
5-fluorouracil (5-FU)	HuR mRNA↑	MDA-MB-231	[77]

Proteins modulating HuR expression

Breast cancer type 1 susceptibility protein (BRCA1)-IRIS is a product of the BRCA1 locus, the expression of which is associated with breast cancer aggressiveness [103]. SiRNA-mediated BRCA1-IRIS silencing and BRCA1-IRIS over expression revealed that BRCA1-IRIS increases total and cytoplasmic, but not nuclear, HuR expression levels in MCF7 and SK-BR-3 cells. The exact mechanism was not elucidated, but it probably involves Nuclear Factor (NF)-kappaB [60], which is known to activate HuR transcription [104]. Moreover, BRCA1-IRIS was shown to increase HuR binding to p53 and PPM1D, enhancing their expression [60].

Cold-inducible RNA-binding protein (CIRBP) is a stress response translation activator, over expressed in breast cancer [105], which may promote cell immortalization. SiRNA-mediated CIRBP silencing and CIRBP over expression revealed that CIRBP positively regulates HuR protein levels and increases HuR-containing cytoplasmic stress granules in MCF7 cells. However, CIRBP induces no changes to HuR mRNA levels or nuclear-to-cytoplasmic ratio [92].

Heat-shock factor protein 1 (HSF-1) is a transcription factor, known to be involved in breast cancer progression and metastasis [106]. SiRNA-mediated HSF-1 silencing down-regulates HuR mRNA and protein levels in MCF7 and Hs578T cells. A reduction of HuR mRNA and protein levels was also noted in a HSF-1 knockdown MCF7 xenograft mouse model. Since HuR mRNA stability is not affected, HSF-1 probably modulates HuR at the level of transcription. In addition, HSF-1 knockdown results in down-regulation of a number of known HuR targets in MCF7 and Hs578T cells, such as HIF-1-alpha, HIF-2-alpha, also known as endothelial PAS domain-containing protein 1 (EPAS-1), VEGF-A, p53, G2/mitotic-specific cyclin-B1 and NAD-dependent protein deacetylase sirtuin-1 (SIRT1) and represses tumour growth and angiogenesis *in vivo* [59].

Tristetraproline (TTP), similar to HuR, is an RNA-binding protein that post-transcriptionally regulates gene expression. TTP binds HuR mRNA in MDA-MB-231 cells, negatively regulating HuR mRNA levels. TTP itself is negatively modulated by miR-29a, which binds to the 3'-UTR of TTP mRNA. An abnormally low TTP:HuR ratio is noted in invasive ductal and lobular carcinomas as compared to normal breast tissue. The low TTP:HuR ratio, together with enhanced miR-29a expression, is also evident in MDA-MB-231 cells in comparison with MCF10A, MCF12A and MCF7 cells. MiR-29a expression in MCF10A cells increases HuR mRNA levels 5-fold, while reducing TTP mRNA levels by 40%, resulting in the abnormally low TTP:HuR ratio. Conversely, miR-29a inhibition in MDA-MB-231 cells decreases HuR mRNA levels by 4-fold, while enhancing TTP mRNA levels 2-fold, and down-regulates HuR targets urokinase-type plasminogen activator (uPa), MMP-1, also known as interstitial collagenase, and MMP-13, also known as collagenase 3, mRNA and protein levels together with cell invasiveness by 64% [49].

MicroRNAs modulating HuR expression

MiR-125 binds to the 3'-UTR (nt 686-692) of HuR mRNA and represses its translation and an inverse correlation was observed between HuR protein level and miR-125 expression levels in MCF10A, MCF7, T47D, SK-BR-3 and HMT-3522-T4-2 cells. MCF10A cells have lower HuR protein levels and higher miR-125 levels than the other cell lines, while high levels of both HuR and miR-125 were noted in MDA-MB-231 cells. MiR-125a and b reduce HuR protein levels in MCF7 and T47D cells, miR-125a slightly decreases HuR protein levels in MCF10A cells, while neither miR-125a nor miR-125b affect HuR protein levels in MDA-MB-231 cells. Further studies revealed that in MCF7 cells, miR-125a represses the HuR target cyclin-E1 expression levels, together with cell proliferation by 72% and migration by 62% in a HuR-dependent manner [54].

MiR-16 was predicted to bind to the 3'-UTR (nt 1881-1901) of HuR mRNA in MDA-MB-231 cells, down-regulating HuR protein but not mRNA levels. As a result, the mRNA levels of HuR target genes, such as COX-2, SIRT1 and c-fos, were also affected. Seventy seven % (10 out of 13) samples derived from invasive ductal breast carcinoma patients exhibited 1.5- and 8.5-fold increases of HuR mRNA and protein levels, respectively in tumour tissues when compared to normal tissues. In contrast, a 37% decrease of miR-16 levels in tumour tissues was noted [63].

Drugs modulating HuR expression and function

Tamoxifen is a drug that targets ER signalling and is widely used in breast cancer treatment. Trichostatin A (TSA) and 5-aza-2'-deoxycytidine (AZA) are inhibitors of histone deacetylation and DNA methylation, respectively. They are used to restore ER expression in ER-negative mammary tumours, sensitizing them to tamoxifen treatment. Surprisingly, they have the opposite effect on ER positive cell lines, reducing ER expression levels [107]. Treatment with AZA (2.5 μM, 96 h) and TSA (100 ng/ml, 16 h) represses mitogen-activated protein kinase (MAPK) 8 phosphorylation and activity, resulting in increased nuclear-to-cytoplasmic HuR expression ratio and decreased ER protein expression in MCF7 cells. Reduced cytoplasmic HuR expression levels were also noted in MDA-MB-231 cells, while total HuR protein levels appear slightly down-regulated in MCF7, but not in MDA-MB-231 cells [37, 76]. In contrast, tamoxifen treatment (2.5 μM) activates MAPK8 increasing cytoplasmic HuR expression levels in MCF7 and MDA-MB-231 cells [37, 102]. Inhibition of MAPK8 in tamoxifen-sensitive MCF7 cells and tamoxifen-resistant BT474 cells resulted in reduced proliferation and increased sensitivity to tamoxifen. SiRNA-mediated HuR silencing in both MCF7 and BT474 cells and HuR over expression in MCF7 cells revealed that HuR plays a role in tamoxifen resistance [37]. Finally, MDA-MB-231 cells were treated simultaneously with AZA, TSA, and tamoxifen. When

tamoxifen is added together with TSA cytoplasmic HuR expression levels are increased, but when they are administered separately cytoplasmic HuR expression levels are reduced [102].
Doxorubicin is a widely used chemotherapeutic agent. Doxorubicin treatment (10 μM) induces HuR phosphorylation and subsequent nucleo cytoplasmic shuttling in MCF7 cells and promotes HuR binding to its targets, such as c-fos, c-myc and SOCS3 [79]. More specifically, doxorubicin activates protein kinase C (PKC) delta, which phosphorylates HuR on serines 221 and 318 [46, 61]. In contrast, HuR protein levels are reduced in doxorubicin-resistant MCF7 and MBA-MD-231 cells and HuR cellular localization is not affected by doxorubicin treatment in MCF7 doxorubicin resistant cells [79]. SiRNA-mediated HuR silencing and inhibition of HuR phosphorylation by the use of rottlerin revealed that doxorubicin-induced apoptosis is HuR-dependent [79].
Lapatinib (GW-572016), an oral dual tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), has proved effective for the treatment of advanced HER2-positive breast cancer patients. In contrast, lapatinib treatment of triple negative EGFR-over expressing breast cancer patients enhances migration and invasion, resulting in a worse clinical outcome [108]. A series of experiments using siRNA-mediated gene silencing revealed that lapatinib treatment (1 μM) of MDA-MB-231 cells down-regulates miR-7, resulting in up-regulation of EGFR mRNA and protein levels. Subsequently, EGFR interacts with HuR and enhances HuR binding to COX-2 mRNA, which is then stabilized. Finally, up-regulated COX-2 expression levels lead to increased migration and invasion of MDA-MB-231 cells, *in vitro* and in orthotopic mice [83].
5‑fluorouracil (5‑FU) is a pyrimidine analogue used as anti-cancer therapeutic agent. 5-FU increases HuR mRNA and protein levels in MDA-MB-231 cells in a dose-dependent manner. Similarly, HuR target FOXO1 expression levels are also up-regulated and HuR-mediated modulation of FOXO1 plays a critical role in 5-FU-induced apoptosis [77].

Clinical significance of HuR expression

Clinical studies in DIC patients revealed a statistically significant association between elevated total HuR expression and advanced tumour histological grade and HER2-negative status [56]. Furthermore, nuclear HuR expression pattern was positively associated with histological grade in invasive breast carcinoma patients [66], while cytoplasmic HuR expression pattern was correlated with advanced patients' age and tumour histological grade in carcinoma cases [65], with increased tumour grade in DCIS [64] and in invasive breast carcinomas [66, 68], with increased histological grade and ductal tumour type in familial non-BRCA1/2 cases [94] and in invasive carcinoma patients receiving NACT [64]. Moreover, it correlated with PR-negative status in DCIS [64], in familial non-

BRCA1/2 cases [94] and in invasive carcinoma patients receiving NACT [68], and with ER-negative status in familial non-BRCA1/2 cases [94] and in invasive carcinoma patients receiving NACT [68]. In contrast, cytoplasmic HuR expression pattern was associated with PR-, ER- and HER2-positive status [65]. With reference to the role of HuR as a prognosticator in breast cancer patients, low total HuR expression was identified as an independent prognostic factor for reduced survival rate in DIC patients [57, 62]. Additionally, high cytoplasmic HuR immunopositivity is an independent prognosticator for reduced survival rate in DIC patients [65, 67], invasive carcinoma patients receiving NACT [68] and in familial non-BRCA1/2 patients [94] (Table 4).

Table 4: Clinical significance of HuR expression in breast cancer patients

Type of neoplasia	Patient samples	HuR Localization		HuR Associations & Prognostic Value			Ref.
		nuclear	cytoplasmic	total	nuclear	cytoplasmic	
ADH	71		35/71 (47%)				[64]
DCIS	74		35/74 (49%)			↑ tumour grade ↑ aggressiveness PR(-)	[64]
DIC	82	63/82 (77%)	38/82 (46%)			↑MDR1 ↑ age ↑ histological grade PR(+) ER(+) HER2(+) ↓ survival[b,c]	[65]
	13			↓ miR-16			[63]
	97			↑ tumour grade HER2(-)			[56]
	89			↑ bioenergetic phenotype ↑ survival[c,d]			[62]
	133	132/133 (100%)	53/133 (40%)			↓ survival[b,c]	[67]
	208	128/208 (61%)	63/208 (30%)		↑ histological grade ↑ COX-1 ↑ COX-2	↑ COX-2	[66]
	623		268/623 (43%)			↑ histological grade[a] ↑ ductal tumour type[a] PR(-)[a] ER(-)[a] ↑ p53 ↓ survival[a,b]	[94]
	143			↑ survival[c,d]			[57]
IC + NACT	139		60/139 (42%)			↑ DIC ↑ tumour grade ↑ histological grade PR(-) ER(-) ↓ survival[b,c]	[68]

[a] in familial non-BRCA1/2 cases
[b] overall survival
[c] disease-free survival
[d] progression-free survival

Abbreviations: ADH – atypical ductal hyperplasia; DCIS – ductal carcinoma *in situ*; DIC – ductal invasive carcinoma; IC – invasive carcinoma; NACT – atypical ductal hyperplasia

Conclusion

In total, HuR has been confirmed to bind to 38 protein-coding mRNAs in mammary cell lines, summarized in Table 2, modulating their expression post-transcriptionally. In the majority of the cases and as anticipated HuR stabilizes the target mRNA. Surprisingly though, CD9 antigen mRNA levels were down-regulated in MDA-MB-231 HuR-over expressing cells and up-regulated following siRNA-mediated HuR silencing [52], indicating that HuR may also exert a destabilizing effect. HuR is also known to affect translation, suppressing Wnt-5a [50], delta Np63 [69], IGF-1-R [55] and BRCA1 [56] protein production. There are also a number of genes modulated by HuR for which the regulatory mechanism has not been elucidated, at least in mammary cell lines. Some of them have been studied extensively in other systems; for example HuR is known to enhance p53 translation in colorectal carcinoma cells [75]. In addition, HuR is responsible for the expression regulation of another seven genes, although direct binding of HuR to the mRNAs was not demonstrated in mammary cell lines. Again, four out of seven of these genes, CCNB1, HIF1, HIF2 and PLAU are known HuR targets, modulated at the mRNA stability level [33, 109-111].

Of particular interest are gene products differentially regulated by HuR in breast cancer cell lines representing various tumour progression stages and HuR appears to exert opposite effects on VEGF-A in MCF7 and MDA-MB-213 cells. More specifically, HuR was observed to up-regulate VEGF-A protein levels in non-metastatic MCF7 cells [59], possibly by stabilizing its mRNA as reported previously [112]. In contrast, in an *in vivo* mouse model xenografted with MDA-MB-231 cells, HuR repressed VEGF-A protein levels [72]. Another gene product differentially regulated in MCF7 and MDA-MB-213 cells is CD9 antigen [52].

The majority of genes modulated by HuR are implicated in biological processes such as cell proliferation, invasion and migration, together with angiogenesis and tumour growth. Additionally, the link between HuR and these processes has been demonstrated directly in some of the studies reported here verifying the importance of HuR expression and function in breast cancer progression (Figure 2). Interestingly, HuR promotes growth in the non-tumorigenic MCF10A cells and in the ER-positive MCF7 cells, as illustrated both by direct proliferation assays [57, 69, 71] and by the regulation of genes involved in cell cycle (Table 2). In contrast, HuR has little effect on the growth of the ER-negative, highly tumorigenic MDA-MB-231 cells; however, it appears to be at least partly responsible for their invasive phenotype [49, 73]. Regarding apoptosis, HuR up-regulates pro-apoptotic genes in MCT-1-transformed MCF10A cells (Table 2), but has no effect on MDA-MB-213 cells [72]. Figure 3 summarises the HuR-mediated differential regulation of important biological processes in different cell lines.

Moreover, a number of clinical studies in breast cancer patients demonstrate that HuR is significantly correlated with advanced clinicopathological parameters, indicating that high HuR expression levels may constitute an aggravating factor for tumour growth and metastasis. Furthermore, a cytoplasmic HuR expression pattern appears to be an independent prognostic factor for reduced breast cancer patients' survival. This observation is in agreement with clinical studies in other cancer types [34].

Since HuR appears to be a common denominator and regulator for a number of pathways crucial for tumour formation, growth and metastasis, is implicated in chemo resistance mechanisms to therapeutic drugs, such as tamoxifen, and is associated with important potential therapeutic targets, such as cyclin D1 [113], CDK1 [114], CDK7 [115], MPP-13 [116], YES1 [117], it is feasible that HuR itself could constitute a possible drug target for cancer therapy. Targeting HuR would likely mitigate the severity of the disease and delay progression and, since HuR is implicated in multiple cancer-related pathways, it should be possible to simultaneously block at least a few if not all of them using therapeutic agents that act *via* HuR. Interestingly, HuR is implicated in retinoic acid-, doxorubicin- and 5-FU-mediated apoptosis [77-79], suggesting another possible mode of action for the prospective therapeutic drugs. Future studies should be focused to the discovery and development of HuR-specific drugs for treatment of breast cancer and possibly other cancer types. Recently a number of high-throughput screening methods have been established to identify low-molecular-weight agents against HuR, including a confocal fluctuation spectroscopic assay [118], fluorescence polarisation assays [119, 120], coupled with nuclear magnetic resonance [120] and a mammalian cell based system [121]. As a result, a number of promising chemicals binding to HuR and disrupting HuR dimerization and HuR-mRNA interactions have been reported, such as MS-444, dehydromutactin, okicenone [118], quercetin, b-40, and b-41 [122], mitoxanthrone [121], CMLD-2 [119] and 15,16-dihydrotanshinone [123]. The latter, a traditional Chinese medicine, is of particular interest since it has been assessed in breast cancer cell lines and was shown to have HuR-dependent anti-proliferative (1 mM) and cytotoxic (10 mM) effects in MCF-7 cells, and to inhibit the migration of MDA-MB-231 cells [123]. Additionally, 15,16-dihydrotanshinone [123] and dehydromutactin [118] were noted to affect the subcellular localisation of HuR, leading to increased nuclear-to-cytoplasmic HuR ratio. Similarly, apoptosis-inducing CMLD-2 was demonstrated to be preferentially cytotoxic against (colon and pancreatic) cancer cells when compared to normal cells and to suppress the oncogenic Wnt signalling [119]. All the above effects are mediated by the resulting alterations to mRNA stability and translation of various HuR target genes. Two more of the aforementioned chemicals, MS-444 and okicenone, were already known for their properties against cancer, which are now shown to be at least partly HuR-dependent [118]. Which, if any, of these compounds would

be effective against breast cancer remains an open question. To this end, further investigation is required in order to elucidate HuR mechanisms of action and determine at which stage of the disease and against which types of breast cancer a specific therapeutic agent targeting HuR would be more effective. The construction of stable mammary cell lines for inducible HuR expression would be an initial step towards this direction, prior to *in vivo* animal and clinical studies.

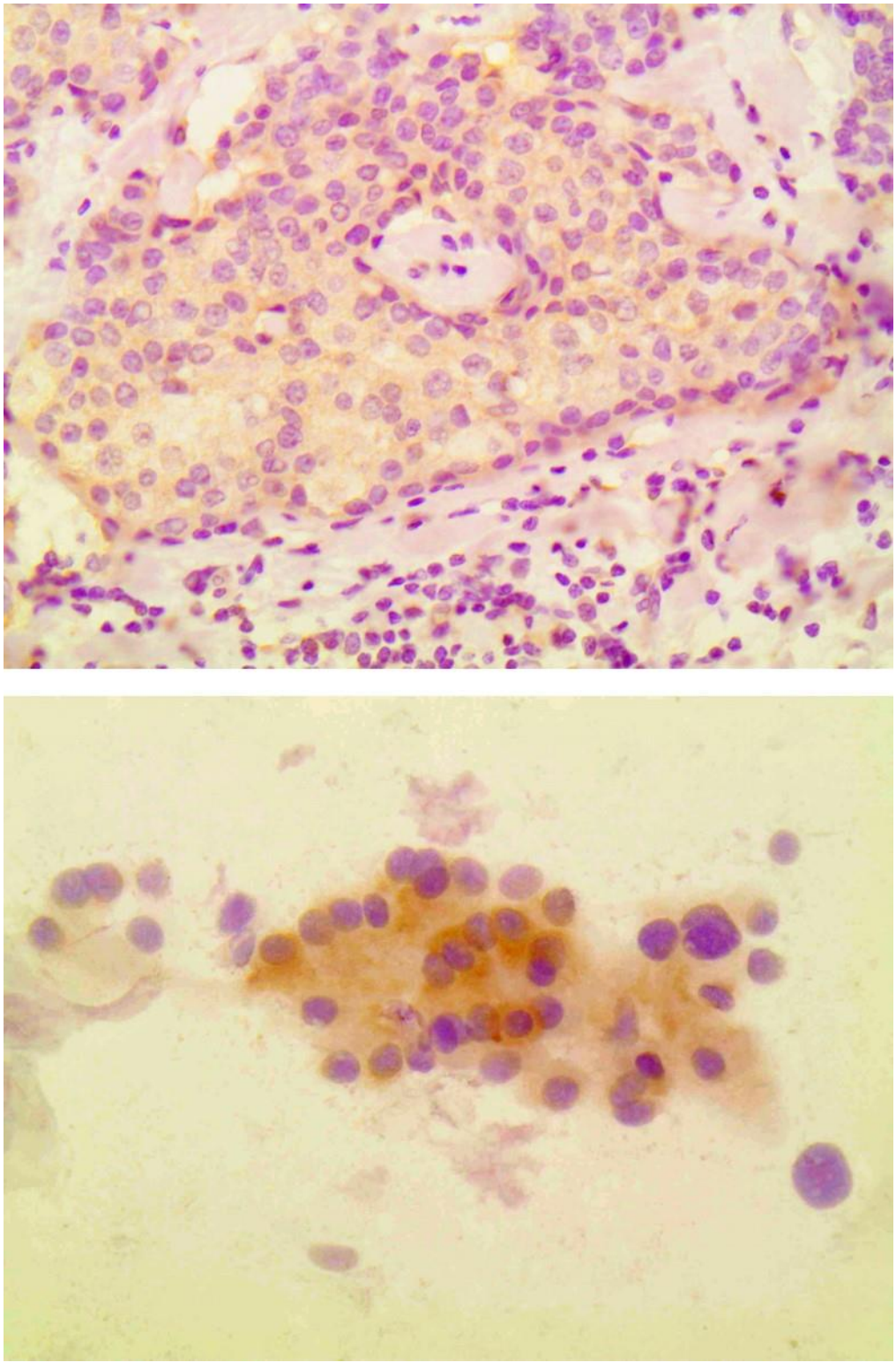

Figure 1: Representative immunostainings for HuR protein expression in histological and cytological samples of invasive breast carcinoma (original magnification 400x).

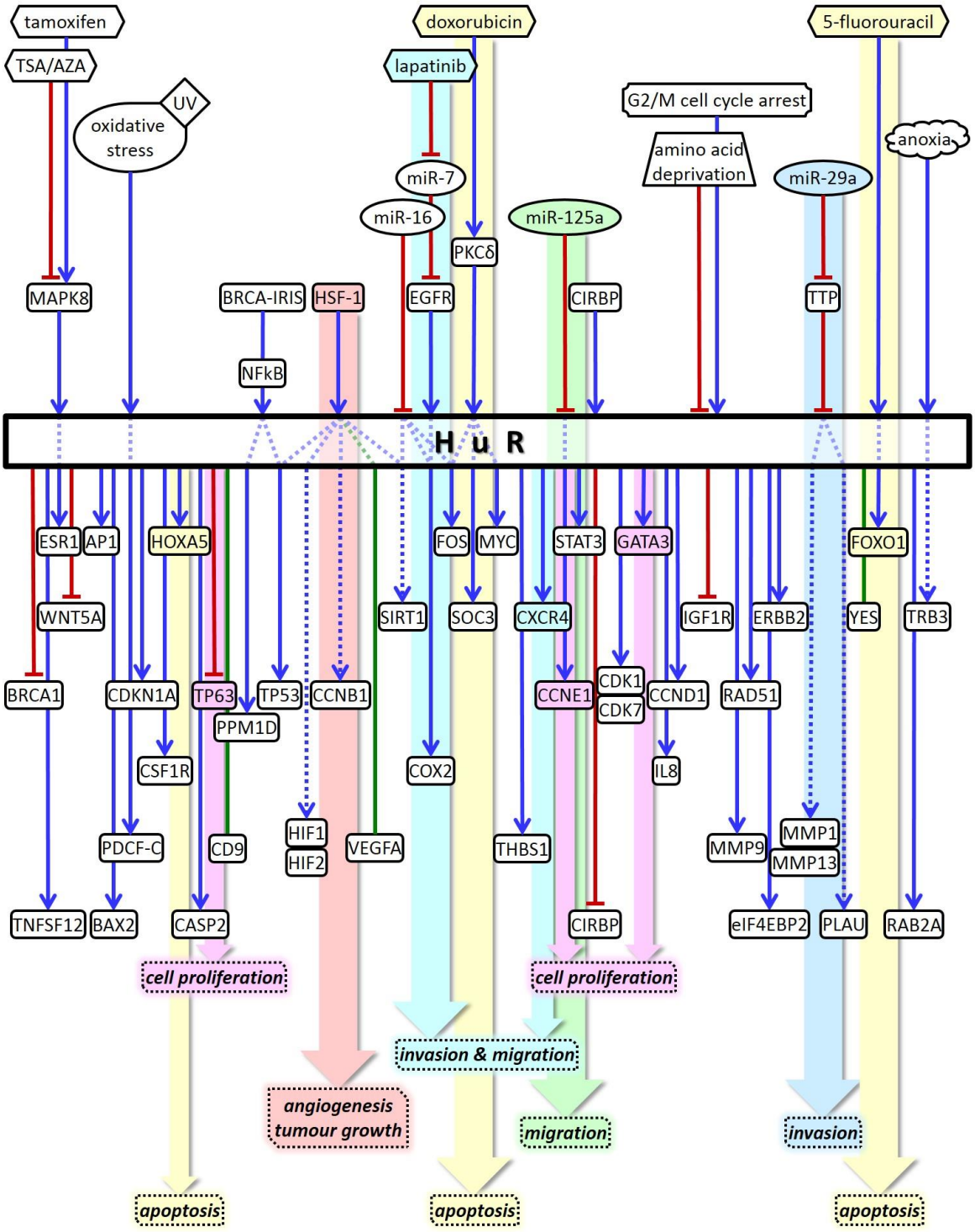

Figure 2: Schematic representation of the regulatory axes mediated by HuR in breast cancer cells. HuR is the wide rectangle in the middle of the cartoon. All shapes above the rectangle are proteins, miRNAs, drugs and environmental conditions regulating HuR expression and function. All shapes below the rectangle are genes regulated by HuR. Blue arrows indicate positive regulation; red horizontal lines indicate negative regulation; dotted lines signify the lack of known direct binding of HuR on the target gene in breast cancer cells. The wide coloured arrows point to biological processes experimentally proven to be affected by these regulatory axes.

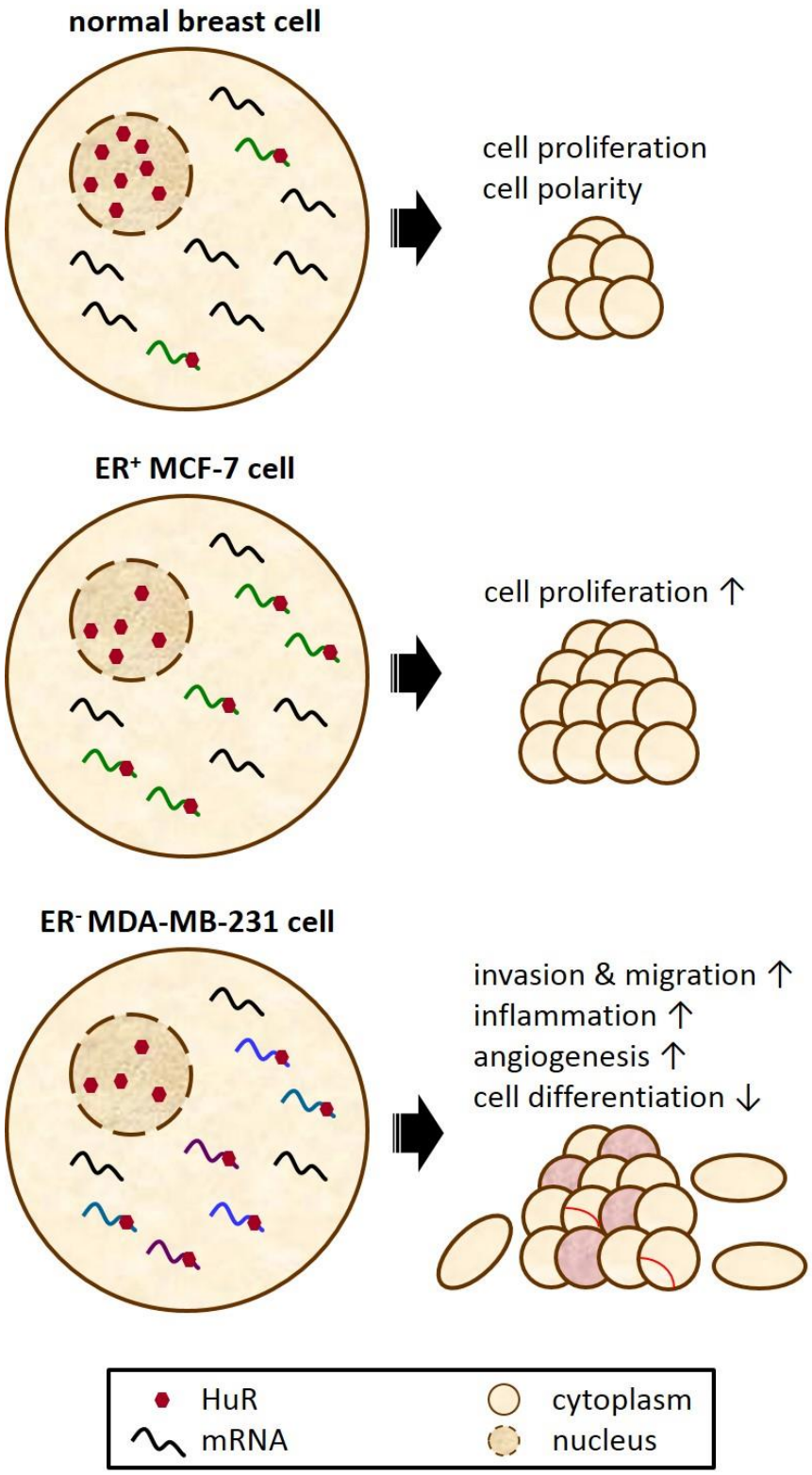

Figure 3: Proposed model on the localisation and function of HuR in normal mammary cells, early-stage mammary tumours (as typified by the ER-positive MCF-7 cell line) and late-stage mammary tumours (as typified by the ER-negative MDA-MB-231 cell line). The cytoplasmic HuR expression levels are correlated with the degree of malignancy and HuR binding to different target mRNAs leads to differential regulation of cancer-related biological processes.

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