
Post-Transcriptional Regulation of Proto-Oncogene *c-fms* in Breast Cancer

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1. Introduction

1.1. *c-fms* and breast cancer

In the development and progression of breast cancers, both the *c-fms* proto-oncogene (which encodes the tyrosine kinase receptor for CSF-1) as well as CSF-1 (colony stimulating factor-1), play an important role. Evidence from transgenic models suggests that *c-fms* encodes for the sole receptor for CSF-1 (Dai *et al*, 2002). We and others have found that *c-fms* and/or CSF-1 are expressed by the tumor epithelium in several human epithelial cancers (Kacinski *et al*, 1988, 1990, 1991; Rettenmier *et al*, 1989; Filderman *et al*, 1992; Ide *et al*, 2002); elevated levels of *c-fms* and CSF-1 are associated with poor prognosis (Kacinski *et al*, 1988; Tang *et al*, 1990; Price *et al*, 1993; Chambers *et al*, 1997, 2009; Scholl *et al*, 1993; Kluger *et al*, 2004; Sapi 2004). In human breast cancer, 94% of *in situ* and invasive lesions express *c-fms* (Kacinski *et al*, 1991; Flick *et al*, 1997), while 36% express both CSF-1 and *c-fms* (Kacinski *et al*, 1991; Scholl *et al*, 1993). Among breast cancer patients, serum levels of CSF-1 are frequently elevated in those with metastases (Kacinski *et al*, 1991). In breast tumors, nuclear CSF-1 staining is associated with poor survival (Scholl *et al*, 1994), and *c-fms* expression confers an increased risk for local relapse (Maher *et al*, 1998). In a large breast cancer tissue array, *c-fms* (Kluger *et al*, 2004) is strongly associated with lymph node metastasis, and poor survival. This strong correlation with prognosis suggests an etiologic role for *c-fms*/CSF-1 in tumor invasion and metastasis.

Tumor-associated macrophages bearing CSF-1 promote progression of breast cancer (Pollard 2004). In mice bearing human breast cancer xenografts, targeting mouse (host) *c-fms* with siRNA, or CSF-1 with antisense, siRNA or antibody suppressed primary tumor growth by 40-50% (Aharinejad *et al*, 2004; Paulus *et al*, 2006), and improved their survival (Aharinejad *et al*, 2004). Hence, paracrine signaling by macrophages bearing CSF-1 also plays a critical role in breast cancer progression. Transgenic models suggest that the absence

of CSF-1 results in delay of tumor invasion and metastasis, while targeting CSF-1 to mammary epithelium in these models enables macrophage infiltration and invasive breast cancer to develop and metastasize (Lin *et al*, 2001).

We have reported that glucocorticoids (GC) up-regulate *c-fms* expression both in breast cancer cells (Kacinski *et al*, 1991; Flick *et al*, 2002; Sapi *et al*, 1995), and in primary organ cultures of breast cancer specimens (Kacinski *et al*, 2001). In a study of 329 breast cancer patients, 52% of the breast cancer tissues had functional glucocorticoid receptor (GR) (Allegra *et al*, 1979). This allows for breast cancer responsiveness to circulating, endogenous GCs.

In the *in vivo* environment, with endogenous GCs, we observed extensive metastatic spread by breast cancer cells over-expressing *c-fms*, compared to controls (Toy *et al*, 2005). Parenchymal invasion was demonstrated only by the *c-fms* overexpressing cells. Interrupting the autocrine loop between *c-fms* and CSF-1 inhibits GC-stimulated invasiveness, motility, and adhesiveness *in vitro* of breast cancer cells (Toy *et al*, 2010). This mechanism of increasing *c-fms* by GC becomes aberrantly up-regulated in invasive, metastatic breast cancer.

1.2. Regulation of *c-fms* expression

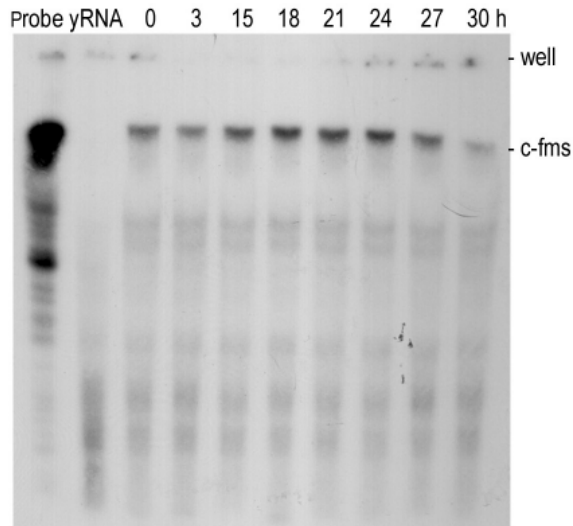
Regulation of *c-fms* expression is a complex process. Both transcriptional and post-transcriptional regulations are involved to maintain a proper level of *c-fms* expression. This chapter summarizes the research over the last 20 years concerning post-transcriptional regulation of *c-fms* and its expression in breast cancer.

1.3. Stability of *c-fms* transcripts in breast cancer cells

c-fms expression is high in metastatic breast cancer cells, but not detectable in the normal breast cells and non-invasive precursors of breast neoplasms (Kacinski *et al*, 1988, 1990). Unusually long half-life of *c-fms* mRNA partially contributes high expression in metastatic breast cancer cells (Chambers *et al*, 1994, Woo *et al*, 2011). GCs increase the *c-fms* mRNA half-life from 9.6 h to 18.9 h in BT20 breast cancer cells (Woo *et al*, 2011). In highly invasive MDA-MB-231 breast cancer cells, *c-fms* mRNA half-life increases up to 27 h in response to GC treatment (Figure 1).

1.4. Post-transcriptional regulation of *c-fms* expression by 3'UTR

mRNA 3'UTR contains *cis*-acting regulatory sequences which are involved in regulation of mRNA stability and polyadenylation (Mignone *et al*, 2003; Bashirullah *et al*, 2001), mRNA degradation (Bevilacqua *et al*, 2003), translation, and subcellular localization of mRNAs (Loya *et al*, 2008; Jansen, 2001). Mutations in 3'UTR could result in diseases and are proposed as 'a molecular hotspot for pathology (Chen *et al*, 2006; Conne *et al*, 2000). Post-transcriptional regulation exerted by 3'UTR is considered an important counterpart to transcriptional regulation for maintaining the proper level of gene products in the cell.



Probe – free probe, yRNA – yeast RNA as negative control, Total RNA was isolated after dexamethasone treatment at the indicated time.

Figure 1. RNase protection analysis of *c-fms* mRNA in MDA-MB-231 cells treated by 400 nM dexamethasone.

Human *c-fms* mRNA 3'UTR encodes 774 nt and contains unique regions including a non-AU-rich-69 nt sequence (3499-3567) which we have described and characterized (Woo *et al*, 2009, 2011), and also several putative target sequences for miRNA binding (Figure 2). The 69 nt sequence contains 3 islets of pyrimidine-rich sequences (CUUU). Mutations in these pyrimidine-rich sequences in 69 nt disrupted vigilin and HuR binding (Woo *et al*, 2009, 2011).

In metazoans, the 69 nt sequence within the 3'-UTR of *c-fms* mRNA is partially conserved between human, mouse, and rat (Figure 2). This region does not contain conventional AU-rich elements (ARE) (Woo *et al*, 2009). Overall, the 69 nt sequence is slightly pyrimidine-rich (>57-61%) and we proposed that primary sequence as well as loop structure may be important for protein binding (Woo *et al*, 2011; Kanamori *et al*, 1998). Indeed, this 69 nt region is predicted to form a stable loop structure (Figure 3).

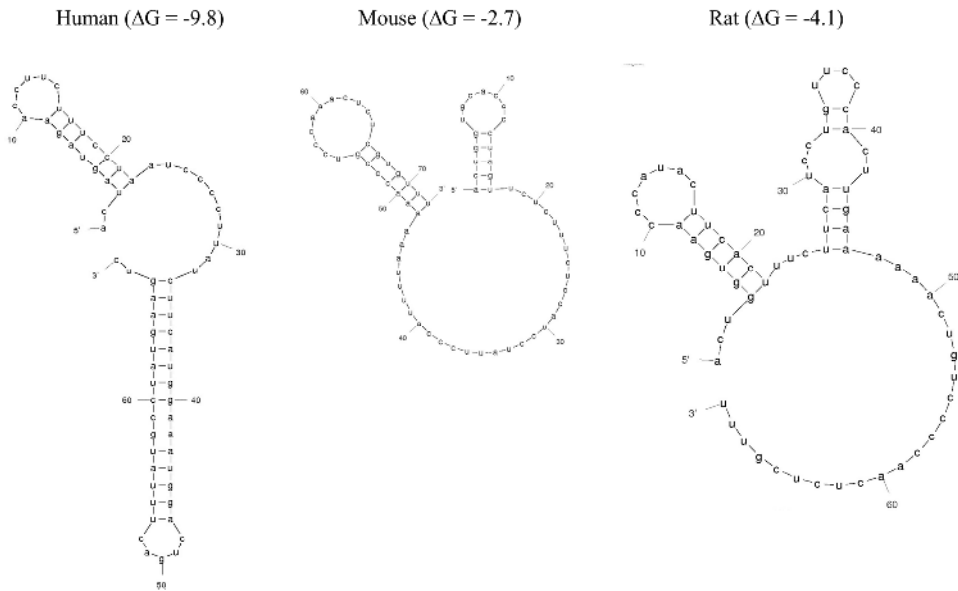


Figure 3. RNA loops of 69 nt are predicted by mfold (<http://mfold.rna.albany.edu/?q=mfold>).

1.5. microRNAs for *c-fms* mRNA regulation

MicroRNAs (miRNAs) are 21-23 nucleotide single-stranded RNAs, that in general down-regulate translation and enhance mRNA degradation (Huntzinger and Izaurralde, 2011; Braun *et al*, 2011). As a consequence, miRNAs are involved in the regulation of several biological functions (differentiation, hematopoiesis, tumorigenesis, apoptosis, development, proliferation, and growth) (Kim, 2005). They are predicted to regulate more than 60% of human mRNA (Friedman *et al*, 2009). It has been found that mRNAs with long 3'UTRs are more susceptible to miRNA regulation than those with short 3'UTRs as the latter lack the number of binding sites necessary for multiple miRNA binding and regulation (Stark *et al*, 2005).

Bioinformatics analysis predicted eight miRNAs (miR-339-5p, miR-449, miR-34, miR-610, miR-22, miR-134, miR-155, and miR-217) targeting six regions in *c-fms* mRNA 3'UTR (Figure 2). These six target regions are also highly conserved in human, mouse and rat. Among those, two miRNAs (miR-610 and miR-155) were selected by us for further analysis. *C-fms* mRNA level is higher in BT20 epithelial breast cancer cells than in Hey epithelial ovarian cancer cells (Figure 4). In contrast, miR-610 and miR-155 RNA levels show opposite expression patterns with their RNA levels lower in BT20 than in Hey cells. Using a luciferase RNA-fused *c-fms* mRNA 3'UTR reporter system, introduction of miR-610 inhibitors in BT20 cells increased luciferase RNA level by 5.5-fold and luciferase activity by 1.3-fold. The down-regulation of mir-610 has more effects on luciferase RNA levels than translational repression. Some reports describe miRNA effects to be mainly on translational

repression, while others describe an effect primarily on mRNA decay. Guo *et al* (2010) reported that the predominant effect of mammalian miRNAs is on mRNA decay which results reduced translation. In contrast, in zebrafish, miR-430 reduced translation initiation prior to inducing mRNA decay (Bazzini *et al*, 2012). Djuranovic *et al* (2012) reported miRNA-mediated translational repression is followed by mRNA deadenylation. Recently, the concept of mRNA destabilization by miRNAs gained support by genome-wide observation studies (Huntzinger and Izaurralde, 2011).

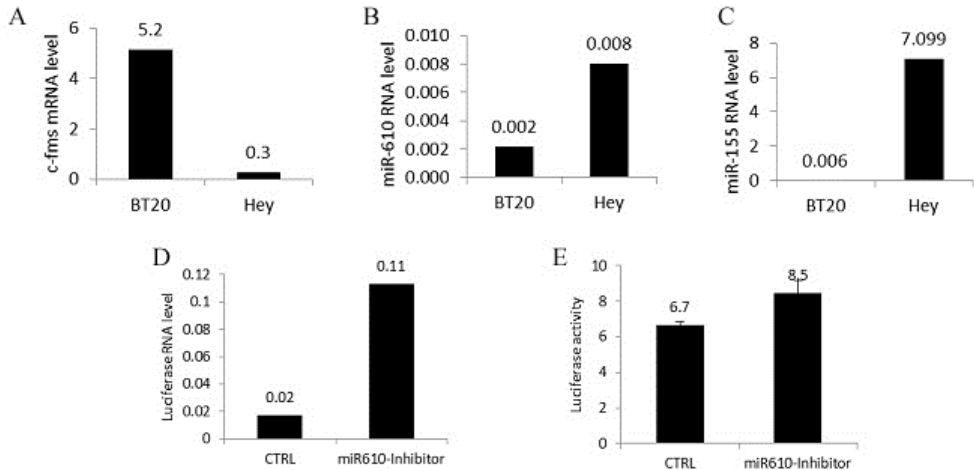


Figure 4. (A) *c-fms* mRNA level is higher in BT20 than in Hey cells. (B) miR-610 RNA level is higher in Hey than in BT20 cells. (C) miR-155 RNA level is higher in Hey than BT20 cells. (D) Using a luciferase RNA-fused *c-fms* mRNA 3'UTR reporter system, introduction of miR-610 inhibitor increased luciferase RNA level by 5.5-fold and (E) luciferase activity by 1.3-fold in BT20 cells.

1.6. RNA-binding proteins for *c-fms* mRNA metabolism and translation

The first evidence supporting post-transcriptional regulation of *c-fms* mRNA by RNA-binding proteins was reported in human monocytes (HL-60 cells) (Weber *et al*, 1989). In their study, TPA (12-*O*-tetradecanoylphorbol-13-acetate)-induced monocytic differentiation did not change *c-fms* transcription, but increased *c-fms* mRNA level. In addition, treatment of protein synthesis inhibitor cycloheximide decreased half-life of *c-fms* mRNA in TPA-induced HL-60 cells. From this observation, they proposed that a labile protein(s) is involved in stabilization of *c-fms* mRNA.

Chambers *et al*. (1993) reported the existence of mRNA regulatory proteins involved in *c-fms* mRNA destabilization in dexamethasone (Dex) or cyclosporin A (CsA) treated HL-60 cells. Dex or CsA blocked TPA-induced monocytic differentiation as well as TPA-induced adherence and further differentiated morphology. In TPA-induced HL-60 cells, *c-fms* mRNA half life was decreased after the addition of Dex or CsA. The effects of cycloheximide of *c-fms* mRNA decay in this setting suggested the existence of labile destabilizing protein(s).

Furthermore, in breast carcinoma cells (BT20 and SKBR3), Dex-treatment at later time points increased *c-fms* mRNA level without affecting *c-fms* transcription. Addition of protein synthesis inhibitors prevented Dex-induced increase of *c-fms* mRNA level suggesting the presence of Dex-inducible stabilizing protein(s) in breast carcinoma cells (Chambers *et al*, 1994).

RNA-binding proteins: About 1,500 RNA-binding proteins (RBPs) have been identified, which bind to mRNA and modulate mRNA stability and translation. mRNA primary sequences as well as loop structures are known to facilitate regulatory protein binding for post-transcriptional regulation.

HuR – HuR, one of the most extensively studied RBPs, encoded by ELAVL1 (embryonic lethal, abnormal vision, *Drosophila*-like 1) binds *cis*-acting AU-rich elements (AREs) (Barreau *et al*, 2005) and also non-ARE-containing sequences including pyrimidine-rich sequences (Woo *et al*, 2009) in target mRNAs. HuR stabilizes and increases half-life of target mRNAs and therefore enhances their translation (Srikantan and Gorospe, 2011). Our study indicates that HuR binds *c-fms* mRNA 3'UTR and enhances mRNA stability and translation (Woo *et al*, 2009).

In human breast-cancer tissues, HuR is expressed mostly in nucleus (>90%), but expression in cytoplasm is also found. High nuclear expression of HuR is a poor prognostic factor both in breast and ovarian cancer (Woo *et al*, 2009; Yi *et al*, 2009).

Vigilin – Vigilin, a high-density lipoprotein-binding protein, contains 15 K-homology (KH) domains (Goolsby and Shapiro, 2003). The KH domain protein family interacts with ARE-containing mRNAs and enhances mRNA degradation and consequently down-regulates

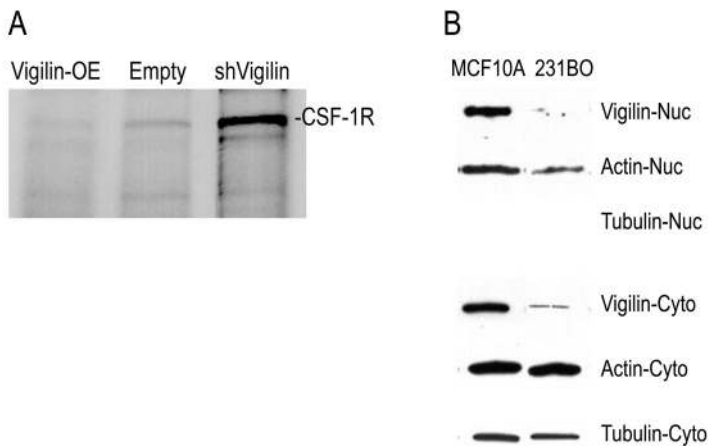


Figure 5. (A) Metabolic labeling and immunoprecipitation of CSF-1R. (B) Immunoblot of Vigilin in both nuclear (Nuc) and cytoplasmic (Cyto) fractions of MCF10A and MDA-MB-231BO cells. Absence of tubulin in nuclear fraction and presence of tubulin in cytoplasmic fraction indicate no cross-contamination in both fractions.

translation (Gherzi *et al*, 2004). In contrast, vigilin interacts largely with unstructured pyrimidine-rich sequences in mRNA 3'UTR (Kanamori *et al*, 1998; Woo *et al*, 2011). We found that vigilin decreases *c-fms* mRNA half-life and down-regulates translation. Ectopic expression of vigilin in breast cancer cells showed that the effects of down-regulation is more pronounced on *c-fms* protein level than on the mRNA level (Woo *et al*, 2011). Metabolic labeling and immunoprecipitation of *c-fms* protein showed that vigilin overexpression down-regulated *c-fms* protein level in BT20 cells (Figure 5A). In contrast, suppression of vigilin by shRNA up-regulated *c-fms* protein level.

Furthermore, immunoblot analysis showed that vigilin expression was lower in metastatic breast cancer MDA-MB-231BO cells than in non-tumorigenic epithelial breast MCF10A cells (Figure 5B). This indicates that a possible suppressive role of vigilin in invasive characters of breast cancer cells.

Both *in vitro* and *in vivo* studies indicate that vigilin and HuR competitively bind to the pyrimidine-rich 69 nt sequence of *c-fms* mRNA 3'UTR (Figure 4, Woo *et al*, 2009, 2011). *In vitro* competition assay showed that affinity of vigilin to the 69 nt sequence is at least 3-fold higher than that of HuR (Figure 6).

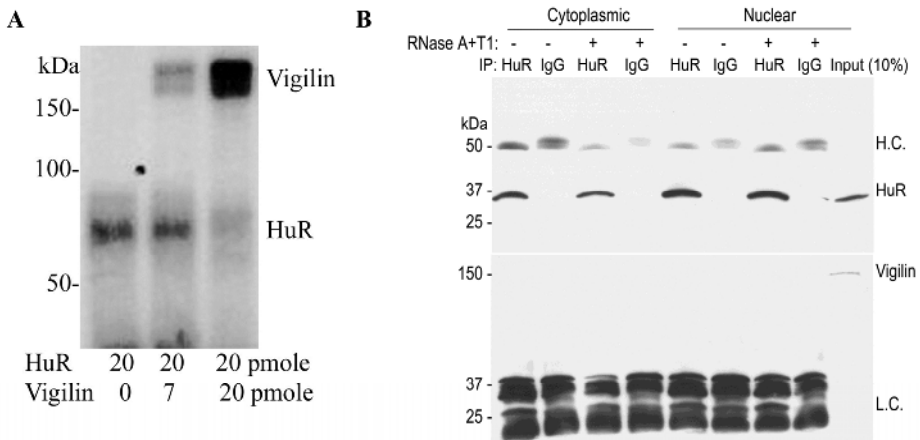


Figure 6. (A) Competition assay between vigilin and HuR by UV crosslink. (B) Co-immunoprecipitation assay. Vigilin and HuR do not present in the same mRNP complexes. IP assays were carried out using cellular lysates from MDA-MB-231 cells in either RNase-free or RNase-treated conditions using anti-human HuR mAb, or IgG. The presence of HuR in the IP materials was monitored by immunoblot. H.C. – heavy chain of IgG. L.C. – Light chain of IgG.

1.7. Effects of HuR and vigilin on invasiveness of breast cancer cells

Increased *c-fms*/CSF-1 levels correlate with the invasive breast cancer phenotype, and with prognosis (Toy, 2005; Toy *et al*, 2010; Sapi, 2004; Kluger *et al*, 2004; Scholl *et al*, 1994, 1993; Maher *et al*, 1998). We studied the ability of BT20 breast cancer cells to invade through a human derived simple matrix *in vitro*. The invasion of BT20 cells was significantly inhibited

by the over-expression of vigilin, resulting in a 48% decrease compared to control (Figure 7). In contrast, over-expression of HuR increased invasiveness by 34%. Our findings suggest that vigilin can negatively impact, through suppression of *c-fms* expression, breast cancer cell invasiveness. In contrast, HuR enhances breast cancer cell invasiveness.

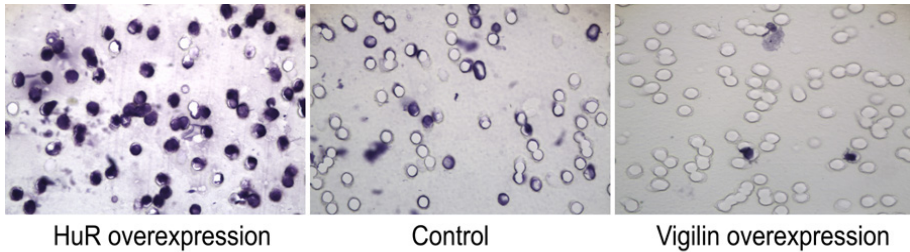


Figure 7. Vigilin and HuR regulate *in vitro* invasiveness of BT20 breast cancer cells. This findings correlate with relative *c-fms* expression.

1.8. Post-translational modification: dimerization and tyrosine-phosphorylation of CSF-1R activation of PIP3/Akt signal transduction pathway

Activation of CSF-1R, product of the *c-fms* gene, requires ligand-induced non-covalent dimerization and phosphorylation of tyrosine residues in CSF-1R (Xiong *et al*, 2011; Li and Stanley, 1991). Here, we focus on one of the major signaling transduction pathways which result from CSF-1R activation. Phosphorylated CSF-1R interacts with PI3K (Phosphatidylinositol 3-kinases) (Shurtleff *et al*, 1990). In turn, PI3K converts PIP2 (Phosphatidylinositol-3,4-bisphosphate) to PIP3 (Phosphatidylinositol-3,4,5-tisphosphate). PIP3 interacts with Akt (protein kinase B, PBK), and activates downstream components in the PIP3/Akt signaling pathway. As a result, several physiological consequences are regulated including cell proliferation, apoptosis, and growth. An activated PIP3/Akt pathway is a common event in human cancer. (Arcaro and Guerreiro, 2007).

In breast cancer cells, multiple components are known to activate phosphorylation of CSF-1R. Endogenous cytokine CSF-1, functioning as an autocrine signal, can bind to the extracellular domain of CSF-1R and activate the cytoplasmic kinase domain leading to autophosphorylation of tyrosine-residues in CSF-1R. There is evidence to suggest that endogenous CSF-1 can also bind CSF-1R without interaction on the membrane surface. Exogenous CSF-1, from other sources such as macrophages, osteoclasts, or fibroblasts, can function in a paracrine manner to activate CSF-1R on the membrane surface. Consequently, phosphorylation of tyrosine residues in CSF-1R activates cell proliferation and invasive potential (Yu *et al*, 2012; Sapi *et al*, 1996). Our study indicates glucocorticoids (dexamethasone) and starvation also activate CSF-1R auto-phosphorylation (Figure 8).

CSF-1R is localized both in the cytoplasm, plasma membrane, and nuclear envelope (Zwaenepoel *et al*, 2012). CSF-1R in the nuclear envelope becomes phosphorylated in response to CSF-1. Phosphorylated CSF-1R in the nuclear envelope triggers the phosphorylation of Akt and p27 inside the nucleus.

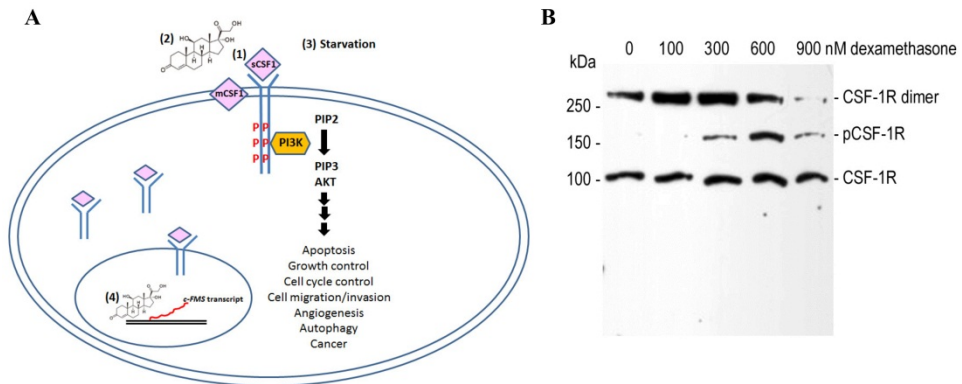


Figure 8. (A) Signal transduction through pCSF-1R/PI3K regulates cell growth and angiogenesis. Both autocrine and paracrine signals (sCSF-1, glucocorticoids, and starvation) trigger dimerization and autophosphorylation of CSF-1R, which interacts with PI3K. The PI3K generates PIP3, which binds to Akt. Activation of PIP3/Akt activates downstream components and regulates growth, apoptosis and cell cycle. (B) Dexamethasone induces autophosphorylation of CSF-1R in starved MDA-MB-231 cells.

2. Discussion

Post-transcriptional and translational regulation of *c-fms* expression by vigilin and HuR in breast cancer cells: mRNA translation and decay are complex multi-staged processes. Mature mRNAs either enter translation or degradation pathways depending on the developmental stages of the cell. We have reported vigilin and HuR, both nuclear-cytoplasmic shuttling RNA-binding proteins, to be involved in post-transcriptional as well as translational regulation of *c-fms* mRNA (Woo *et al*, 2009, 2011). Vigilin binds the pyrimidine-rich 69 nt sequence in the *c-fms* mRNA 3'UTR, to which HuR also binds. Both *in vitro* and *in cell* studies indicate that they compete for the same 69 nt sequence in the *c-fms* mRNA 3'UTR and that dynamic changes in the ratio of vigilin to HuR can influence their ability to associate with the *c-fms* mRNA and post-transcriptionally regulate cellular *c-fms* levels. While vigilin down-regulates *c-fms* translation as well as mRNA stability, HuR, in contrast, has opposite effect on *c-fms* levels; i.e., HuR up-regulates *c-fms* mRNA stability resulting increased *c-fms* protein levels. In our previous study, the polysome profile indicates vigilin is associated with free mRNPs and low MW monosomes. In contrast, HuR was detected with high MW polysomes (Woo *et al*, 2011). Vigilin also represses translation of reporter RNA (luciferase RNA fused with *c-fms* mRNA 3'UTR sequence) in the rabbit reticulocyte lysate cell-free translation system (Woo *et al*, 2011).

Translation can be divided in three phases; initiation, elongation, and termination. Translation initiation is a complicated process for which a large number of eukaryotic initiation factors (eIFs) have been identified (Sonnenberg and Hinnebusch, 2009). Translation initiation starts with the assembly of a 48S quaternary initiation complex comprised of the 40S ribosomal subunit, eIFs, tRNA_{Met}, and m⁷G cap of the mRNA. In general, this 48S initiation complex scans and base pairs with the AUG initiation codon in

5'UTR of mRNA. This results in formation of the 80S ribosome and is continued in the elongation step of peptide synthesis.

In a 'closed-loop' mRNP model for cap-dependent translational regulation, PABPs bind both to the poly A⁺ tail at the 3'UTR and eIF4G of the translation initiation complex at the 5'-cap (Huntzinger and Izaurralde, 2011). This mRNA circularization attracts ribosomes to form a translation initiation complex. Subsequently, after translation termination, joining of the 5'- and 3'-ends of the mRNA facilitates the transfer of ribosomal subunits from the 3' to the 5'-end.

Our results have demonstrated presence of vigilin in free mRNP fractions in human BT20 breast cancer cells. While vigilin association with free mRNPs may prevent 'closed-loop' formation and consequently inhibit *c-fms* protein translation, it was also found to associate with tRNAs and elongation factors (Kruse *et al*, 2003; Vollbrandt *et al*, 2004). Binding of vigilin with these components may deplete the available tRNAs and elongation factors for translation elongation. We propose a model that the impaired translation resulting from vigilin binding may expose both 5'- and 3'-ends of the mRNA through reduced circularization and increase its rate of degradation (Figure 9). In contrast, we propose that HuR binding to *c-fms* mRNA 3'UTR may enhance 'closed-loop' formation which increases the *c-fms* mRNA stability and also translation initiation efficiency. Immunoblot analysis indicates that vigilin is, in general, less expressed in breast cancer cells than in non-tumorigenic breast cells (Woo *et al*, 2011). This indicates that down-regulation of vigilin may be partly responsible for increased *c-fms* level in breast cancer cells. In summary, RNA binding proteins, such as vigilin and HuR are critical regulators for determining the fate of proto-oncogene *c-fms* mRNA, either to be translated or decayed.

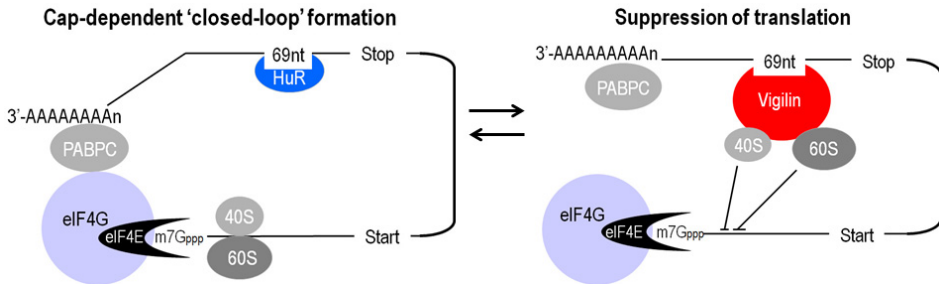


Figure 9. Competition between HuR and vigilin for binding 69 nt of *c-fms* mRNA 3'UTR regulates translational machinery formation. Binding of HuR to 69 nt may induce 'closed-loop' formation. In contrast, binding of vigilin to 69 nt could prevent 'closed-loop' formation.

Future research in post-transcriptional and translational regulation of *c-fms* in breast cancer:

Translational inhibition and mRNA degradation are coordinated processes in which translation initiation is inhibited and translation factors (eIFs) are exchanged with repression/degradation complex (hDcp1/2, Hedls) (Fenger *et al*, 2005), resulting in mRNA degradation by exonucleases (Xrn1 and exosomes) (Balagopal and Parker, 2009). In general, 3'-deadenylation leads to 5'-decapping followed by exonucleolytic digestion at either ends

of mammalian poly-A⁺-mRNAs (Franks and Lykke-Anderson, 2008; Zheng *et al.*, 2008). In human cells, deadenylation is initiated by deadenylase complex (Pan2/3, Caf1, and Ccr4) (Zheng *et al.*, 2008). Deadenylated oligo(A) mRNPs are further processed by decapping complex (including Xrn1 for 5'-to-3' decay) or exosomes (for 3'-to-5' decay). In yeast, decapping activators (Dhh1, Pat1, Lsm1-7, Edc1-3, Scd6) were identified which enhance decapping (Nissan *et al.*, 2010). Mutated or excess nontranslating mRNAs are stored and degraded in processing bodies (P-bodies, GW-bodies, or Dcp-bodies) and/or stress granules (SGs). During inhibition of translation initiation, elevated numbers of P-bodies and SGs are observed (Shyu *et al.*, 2008). Nontranslating mRNPs accumulate both in P-bodies and SGs. Decapping complex (hDcp1/2, Hedls) and mRNA decay fragments are found in P-bodies suggesting presence of 5'-to-3' exonuclease activities (Xrn1). Deadenylation complex (Pan2/3, Caf1, Ccr4) is also present in mouse P-bodies. On the other hand, translation initiation components (eIFs) and RNA-binding proteins (Ataxin-2, Pab1, TIA-R, TIA-1) are found in SGs (Buchan and Parker, 2009). Another very important aspect of mRNA stability is mRNA binding proteins. They can stimulate decapping and degradation processes. Over-expression of cold-inducible RNA-binding protein (CIRP), which represses translation, induces SGs (De Leeuw *et al.*, 2007). In contrast, HuR was shown to release translational repression by helping human mRNA associated with P-bodies to re-enter polysomes (Bhattacharyya *et al.*, 2006). In mammalian cells, P-bodies and SGs often dock together during translation inhibition. Since vigilin was shown to repress *c-fms* translation, it is crucial to understand mechanisms of transitions of *c-fms* mRNPs between P-bodies, SGs and

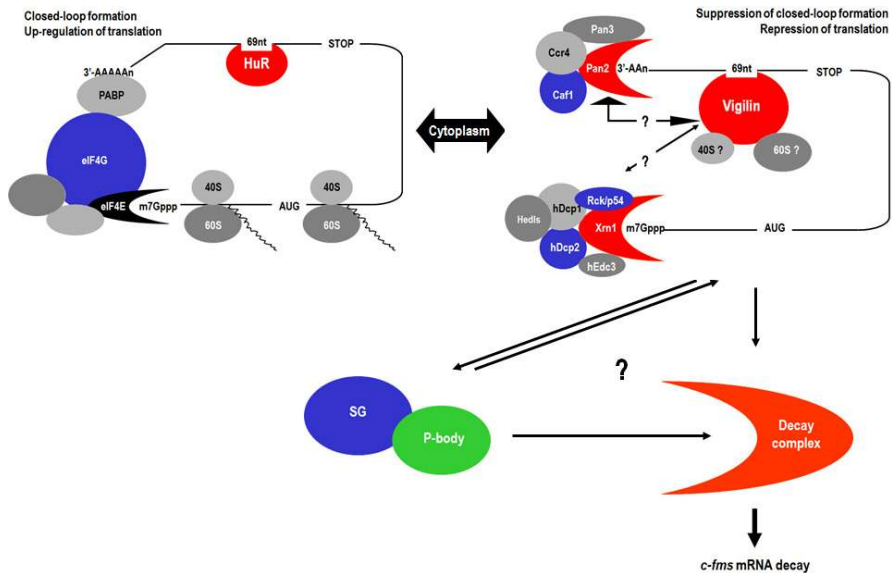


Figure 10. Proposed model for post-transcriptional regulation of *c-fms* by HuR and vigilin. HuR enhances closed-loop formation and increases *c-fms* mRNA stability and translation. In contrast, vigilin prevent closed-loop formation and attracts mRNA degradation complex and down-regulates translation. SG – stress granule

polysomes. A model for these mechanisms is proposed in Figure 10. Elucidating the molecular mechanisms of these exchanges from one state to another is critical to the understanding of regulation of *c-fms* protein levels in breast cancer.

3. Conclusion

In the design of clinical therapeutics, suppression of pathogenic gene expression requires high specificity to prevent off-target toxicity. In order to achieve this, detailed regulatory mechanisms of target gene expression should be elucidated. Understanding the regulatory mechanisms and specific proteins through which vigilin effects translational down-regulation of proto-oncogene *c-fms* in breast cancer can result in more accurate control of its expression.

Based on information available from the last 20 years of research and our recent data, it is now possible to elucidate vigilin's role in translational down-regulation of *c-fms* mRNA in breast cancer. Information obtained from this research will support a model on the manner in which interaction between a specific mRNA (*c-fms*) and proteins (vigilin and HuR) regulates *c-fms* at a translational level. These findings will bring us one step closer to development of a targeted therapy based on these mechanisms.

4. Methods

4.1. Cell culture

A human breast carcinoma cell line BT20 was maintained in MEM (Sigma) supplemented with 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum (Invitrogen) in 5% CO₂ at 37°C. A human breast carcinoma cell line MDA-MB-231 was cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum. For studies using glucocorticoids, cells were grown in starvation medium with 100 nM Dex (Sigma-Aldrich) for 72 h and collected for immunoblot analysis. A human ovarian cancer cell line Hey was grown in DMEM/F12 (Sigma) supplemented with 10% fetal bovine serum.

4.2. Total RNA isolation for semi-quantitative real-time RT-PCR analysis

Cells were grown in 6-well plate for 2-3 days before harvesting. Total RNA was extracted with 500 µl Trizol (Invitrogen) per well. After Trizol extraction, 150 µl of supernatant was carefully removed to avoid genomic DNA contamination. Supernatant was re-extracted by equal volume of chloroform and 100 µl of supernatant was carefully removed and ethanol precipitated for cDNA synthesis.

4.3. Semi-quantitative real-time RT-PCR analysis for *c-fms* mRNAs

Total RNA was oligo-dT₁₈ primed by M-MuLV reverse transcriptase (New England Biolab). For PCR analysis, reverse transcriptase reaction was diluted by 10-fold and 2 µl was used for

20 ul PCR reaction. GAPDH mRNA was amplified in PCR reaction as internal loading control.

c-fms PCR primers (forward primer = 5'-GGAGTTGACGACAGGGAGTACCAC-3', reverse primer = 5'-ACGAGGCCAACACCATGAGAACAG-3').

GAPDH PCR primers (forward primer = 5'-CGGGAAACTGTGGCGTGATGGC-3', reverse primer = 5'-AGGAGACCACCTGGTGCTCAGTG-3').

c-fms mRNA expression level was calculated with the $\Delta\Delta C_T$ method (Schmittgen and Livak, 2008).

4.4. Stem-loop real-time RT-PCR analysis for miR-610 and miR-155 quantification

miRNA expression was determined by the stem-loop qRT-PCR analysis to increase the specificity of miRNA amplification (Chen *et al*, 2005). cDNAs for miR-610, miR-155, and tRNA_{Glu} specific were synthesized using sequence specific stem-loop forming primers. After 10-fold dilution of reverse transcriptase reaction, 2 ul was used for 20 ul real-time PCR. tRNA_{Glu} was used as internal loading control.

miR-610 reverse transcription primer = 5'-gtcgtatccagtgcagggtccgaggtattcgact ggatacactcccag-3')

miR-610 PCR primers (forward primer = 5'-GGCGCTGAGCTAAATGTGTGC-3', reverse primer = 5'-GTGCAGGGTCCGAGGT-3')

miR-155 reverse transcription primer = 5'- gtcgtatccagtgcagggtccgaggtattcgact ggatacagaccct-3'

miR-155 PCR primers (forward primer = 5'-GGCGCTTAATGCTAATCGTGATAG-3', reverse primer = 5'-GTGCAGGGTCCGAGGT-3')

tRNA_{Glu} reverse transcription primer = 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATACGAC GGTGAAAG-3'

tRNA_{Glu} PCR primers (forward primer = 5'-CTGGTAGTACTTGGACGGGAGAC -3', reverse primer = 5'-gtcagggtccgaggt -3')

4.5. Analysis of *c-fms* mRNA Half Life

The *c-fms* mRNA half-life was determined by RNase protection assay (RPA) (Bordonaro *et al*, 1994). Radioactive-labeled antisense RNA probes of *c-fms* mRNA was generated by *in vitro* transcription. *c-fms* cDNA (237nt, 1789-2025) with 67nt random sequence and 23nt T7 promoter at 3'-end was generated by PCR and used as a template for *in vitro* transcription. Probes with specific activity of 1×10^5 cpm were hybridized with 10 μ g of total RNA in hybridization buffer (80% deionized formamide, 40 mM PIPES pH6.4, 400 mM NaCl, and 1 mM EDTA) at 42°C overnight. Next morning, unbound RNA was digested by RNase A and

T1 at 37°C for 1 h. After proteinase K treatment at 37°C for 30 min, samples were extracted by phenol-chloroform and precipitated in ethanol. Samples were analyzed on a 5% acrylamide/8M urea gel and exposed on X-ray film.

4.6. Metabolic labeling and immunoprecipitation of *c-fms* proteins

The BT20 cultures at 75-80% confluence were washed with PBS and incubated in labeling medium (Met,Cys-free RPMI1640 (Sigma R-7513), 5% dialyzed FCS, 500ug/ml Glutamine) for 40 min to deplete endogenous methionine and cysteine in cell. For metabolic labeling, 5 ml labeling medium and 50 ul (500 uCi) of 35S-Methionine/35S-Cysteine per T75 flask was added and incubated for 30-40 min. After brief chase in chase medium (labeling medium with 500µg/ml Cysteine-HCl and 100µg/ml Methionine), cells were harvested and lysed in IP buffer (1% Triton x-100, 0.05% NP-40 in TBS, protease inhibitors). For immunoprecipitation of *c-fms* proteins, 5 ug of *c-fms* monoclonal antibody and 50 µl of Protein A/G-agarose (50% slurry) (Santa Cruz) were added to cell lysates and incubated overnight at 4°C. Next morning, agarose beads was washed extensively with IP buffer and protein was eluted by SDS sample buffer. Labeled protein was analyzed in 10% SDS-PAGE.

4.7. Gain-of-function and loss-of-function assay

Plasmids encoding a control shRNA or shRNA directed against vigilin were purchased from Origene. The shRNAs correspond to coding region nucleotides 614–642 (5'-AAGCTCG GAAGGACATTGTTGCTAGACTG-3') and 829–863 (5'-CATGAAGTCTTACTCATCTCTG CCGAGCAGGACAA-3'), respectively, of human vigilin (GenBank BC001179). An shRNA containing a non-specific 29nt GFP sequence (TR30003, Origene) was used as a transfection control (Empty). For RNAi, 5 ×10⁶ cells were transfected with 10 µg shRNA plasmid using Fugene HD (Roche) according to the manufacturer's instructions. Transfected cells were maintained in culture medium for 3-4 days to permit knockdown before assays.

For vigilin overexpression, pTetCMV-Fo(AS)-vigilin (Cunningham et al, 2000) was transfected using Fugene HD (Roche). The BT20 cells at 75-80% confluence in 6-well plates were transfected with 5 µg of plasmids. The overexpression effects were monitored for 3-4 days by qRT-PCR and western blot analyses.

4.8. UV crosslinking and label transfer with *c-fms* mRNA 3'UTR

UV cross-linking of HuR and vigilin was performed as described previously (Urlaub *et al*, 2000) with modifications. RNAs of *c-fms* 3'UTR labeled with 32P-UTP were incubated with recombinant HuR or recombinant vigilin proteins. The 15 µl reaction mixture contained 5 mM HEPESpH7.6, 1.25 mM MgCl₂, 3.8% glycerol, 0.02 mM DTT, 1 mM EDTA, 25 mM KCl, 50 ng yeast tRNA, 50 ng heparin, 1 mM ATP, and 32P-labeled RNA probe (50,000 cpm). After incubation at 30°C for 20 min, reaction mixture in a 96-well polystyrene plate on ice was illuminated at 254 nm, 125 mJoule for 120 seconds using a GS Gene Linker UV Chamber (Bio-Rad). After crosslink, excess RNA was digested by RNase A for 30 min at 37°C. Crosslinked protein was fractionated in 10% SDS-PAGE.

4.9. Invasion assay

The Membrane Invasion Culture System (MICS chamber) was used to quantitate, the degree of invasion of MDA-MB-231 transiently transfected vigilin or HuR overexpressing clones. Breast cancer cells were cultured in the presence of 100 nM Dex and remained under starved conditions for transfection duration prior to the invasion assays. Parent or transfected cells, 1×10^5 per well in a 6-well plate, were seeded onto 10- μ m pore filters coated with a human defined matrix containing 50 μ g/ml human laminin, 50 μ g/ml human collagen IV, and 2 mg/ml gelatin in 10 mM acetic acid.

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