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Role of CSF-1 in progression of epithelial ovarian cancer

Setsuko K Chambers

Women's Cancers, Arizona Cancer Center, University of Arizona, 1515 N. Campbell Ave., Tucson, AZ 85724, USA, Tel.: +1 520 626 0950, Fax: +1 520 626 8574, schambers@azcc.arizona.edu

Abstract

Despite the dismal outcome seen in the majority of epithelial ovarian cancer patients, there is ongoing progress in understanding the disease at a molecular level. Elucidation of pathways underlying disease progression and metastasis of ovarian cancer is key to development of targeted therapeutics. It is only in this way that therapeutic potential can be translated to reality. Here, we describe the evidence to date for the role of CSF-1/*c-fms* signaling in ovarian cancer invasiveness and metastasis, including the recent understanding of how CSF-1/*c-fms* expression is regulated with identification of significant post-transcriptional regulators.

Keywords

c-fms proto-oncogene; CSF-1; epithelial ovarian cancer; HuR; invasion; metastasis; post-transcriptional regulation; urokinase

Dismal outcome of epithelial ovarian cancer: recent advances in understanding its biology

Epithelial ovarian cancer patients invariably still present with widespread disease throughout the peritoneal cavity, accompanied by invasion into lymphatic channels, and have a poor long-term outcome. Over the last 30 years, there has been no significant improvement in the dismal 15% 10-year survival rate. Ovarian cancer remains the leading cause of gynecologic cancer death among developed nations worldwide [1], and is the fifth leading cause of overall cancer mortality among women in the USA [2]. Barriers to developing effective screening programs include the relatively low prevalence, lack of specific clinical symptoms and absence of a well-defined molecular precursor to elucidate the cell of epithelial cancer origin. Thus, tools for early diagnosis are still in development.

There are three areas of recent advance in understanding the molecular basis for epithelial ovarian cancer. One is in the area of the hereditary epithelial ovarian cancer syndromes, primarily related to germline *BRCA1/2* mutations, which is the genetic basis underlying up to 10% of epithelial ovarian cancer cases. The remaining 90% of cases are sporadic without a clear genetic basis. The second area of advance is in the understanding that epithelial ovarian cancer is not one cancer but a heterogeneous disease. Ovarian cancer has been believed to be the result of an accumulation of genetic alterations. Therefore, ongoing work in the area of treatment and prognostics has focused on targeting specific pathways related to the genes

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thought to be involved in the carcinogenic/metastatic process. Increasingly, evidence supports a two-pathway model of ovarian carcinogenesis [3,4]. This model differentiates between low- and high-grade pathways that are characterized by either a stepwise mutation process (low-grade) or by greater nonstepwise genetic instability that leads to rapid metastasis (high-grade) [3], with different gene-expression patterns observed between the low- and high-grade carcinomas [4]. Furthermore, epithelial ovarian cancer comprises several different histologic subtypes, and some of them, such as the mucinous tumor types, have been found to have distinct molecular profiles from the more common serous histologic subtype. The third area of advance is the discovery that the fallopian tube may also be an important precursor to the high-grade epithelial ovarian cancers, which have a primarily serous histology [5–7]. These lines of evidence suggest that no single approach to targeted therapy or molecularly targeted prognostic factors may be valid across all epithelial ovarian carcinomas.

In comparison with some other malignancies, in which molecular approaches to therapy have revealed both insights and therapeutic advances, at this time the diagnosis of epithelial ovarian cancer usually relegates the patient to a series of toxic therapies that prolong the lifespan by months, not years. While there have been advances in understanding the molecular factors that are associated with epithelial ovarian cancer development and progression, further work is still needed to understand the molecular biology underlying sporadic ovarian cancers and thus impact on the outcome of the majority of ovarian cancer patients.

Introduction to CSF-1 & its receptor encoded by the *c-fms* proto-oncogene

One of the primary roles for the macrophage colony-stimulating factor (CSF-1) is to promote the differentiation and survival of macrophages. The *c-fms* proto-oncogene encodes for the tyrosine kinase receptor for CSF-1. Osteoclasts, which derive from the same hematopoietic precursor as the macrophage, are similarly dependent on CSF-1 for differentiation, activation and survival [8]. CSF-1 also plays an important role in lactation, ovulation, preimplantation, placental function with trophoblastic invasion and regulation of the estrous cycle [9–11]. Thus, as expected, the CSF-1 *op/op* mouse (null mutant for CSF-1) displays, among other phenotypes, impaired mammary development and impaired reproductive phenotype, with a near-absence of tissue macrophages, along with a severe deficiency in osteoclasts, and hence develops osteopetrosis (very dense bones) [12]. Introduction of a transgene for secreted CSF-1 in the *op/op* mouse restores some populations of macrophages, resulting in normal bone, reproductive and neurologic development [13]. *c-fms* is the only receptor for CSF-1, as the same phenotype of osteopetrosis, with near-absence of tissue macrophages and osteoclasts, was observed in a transgenic mouse model nullizygous for *c-fms*, as was observed in the *op/op* mice mutant for CSF-1 [14].

The monocytic lineage expresses *c-fms* and is differentiated and activated into the mature macrophage by CSF-1, as is the osteoclast lineage (Figure 1). How epithelial cancer cells are activated by CSF-1 into a more invasive and metastatic phenotype, which parallels the known activation of macrophages and osteoclasts by CSF-1 is described below.

CSF-1/*c-fms* expression in epithelial ovarian cancer & its role as a biomarker & prognostic factor in this disease

Genes that have been studied in epithelial ovarian cancer are many and include oncogenes, including *HER2/neu*, *c-myc* and *c-fms*, tumor suppressor genes, such as *p53*, *BRCA1* and *BRCA2*, and the growth and other regulatory factors involved in their signaling, including *VEGF*, *LPA*, *IL-6*, *uPA*, and the macrophage colony stimulating factor, *CSF-1* [15].

In addition to the well-recognized roles of CSF-1/*c-fms* in macrophage function [16], trophoblastic invasion [17] and osteoclast formation [18], CSF-1 and/or its receptor have been found to be expressed by the tumor epithelium in several human epithelial cancers, including those of breast, ovarian, lung, endometrial, trophoblastic and prostatic origin [19–25]. In addition, *c-fms*/CSF-1 may also have a role in tumorigenesis of the less common nonepithelial ovarian cancer, the ovarian granulosa cell tumor [26]. In several epithelial cancers, elevated levels of CSF-1 and *c-fms* are associated with poor prognosis [22,27–33].

In normal ovarian surface epithelium, or benign epithelial ovarian tumors [21,34], there is no detectable *c-fms* and only small amounts of CSF-1. We have also previously shown that low malignant potential ovarian tumors minimally express CSF-1 protein [21], and that no tumor of low malignant potential expressed both CSF-1 and its receptor, *c-fms* [27]. Similarly, there was little to no detectable *c-fms* RNA or protein in normal, benign or low malignant potential ovarian tumors [21,34].

In human epithelial ovarian cancer [23,27,31], CSF-1 and/or *c-fms* expression has been observed in the large majority of cases, with 75% of primary tumors and 69% of the metastases expressing CSF-1, and 92% of primary tumors and 83% of metastases expressing *c-fms*. Strong co-expression of CSF-1 and its receptor by ovarian cancer metastases was an independent ($p = 0.007$) poor prognostic factor (relative risk of 2.3-fold). The mean time to recurrence was shortened by 11 months, from 24.1 ± 3.9 months to 13.5 ± 4.0 months, by such co-expression [27]. This co-expression allows for autocrine interaction between cytokine and receptor. We recently uncovered the biological basis for the association between co-expression of CSF-1 and *c-fms* in the metastasis and poor prognosis. We showed that binding of the growth factor, CSF-1, to its receptor (*c-fms* proto-oncogene) activates an autocrine pathway that imparts the invasive metastatic phenotype in this disease [35]. No such co-expression of CSF-1 and its receptor was observed in any of the tumors of low malignant potential, which, by definition, are noninvasive. Among the invasive cancers, no association was found between co-expression of CSF-1 and its receptor and histologic subtype or grade, although the majority of tumors in this cohort were grade 3, serous tumors [27].

Further study was carried out of the role of the activated form of *c-fms* in epithelial ovarian cancer [31]. With CSF-1 activation, phosphorylation of several tyrosine kinase residues in the *c-fms* protein result in downstream signal transduction. Expression of two major autophosphorylation sites (tyrosine 809 and 723) were studied with antibodies specifically produced to recognize these sites. The roles of these two activated tyrosine sites in invasiveness and metastatic potential have been previously characterized in breast cancer cells [36]. Expression of these activated forms of *c-fms* was common, and found to be present in nearly half of both primary and metastatic ovarian cancer specimens. In addition, in the metastases, co-expression of the phosphorylated tyrosine 723 form along with *c-fms* (representing other activated forms of *c-fms* as well as resting *c-fms*) portend a significant decrease in survival ($p = 0.0007$) with increased risk of recurrence [31].

At the time of diagnosis in epithelial ovarian cancer patients, elevated levels of both serum and ascitic fluid CSF-1 are associated with a poor outcome [29,30]. During the course of the disease, elevated levels of serum CSF-1 can herald disease recurrence or progression [37]. Elevated CSF-1 levels as part of a panel of markers, including CA125, has recently been shown to help improve early detection of ovarian cancer [38,39]. This strong association with disease detection and prognosis suggests an etiological role for *c-fms*/CSF-1 in ovarian cancer initiation and neoplastic progression.

CSF-1/*c-fms* in other cancers

Among other epithelial cancers, the role of CSF-1/*c-fms* has been most extensively studied in breast cancer. Similar findings as described for ovarian cancer were observed in the studies of the role of CSF-1/*c-fms* in human breast cancer progression [23,28,32,33]. Normal resting breast tissue (in the absence of pregnancy) does not express *c-fms*, with small levels of detectable CSF-1 [11,23]. A total of 94% of *in situ* and invasive lesions express *c-fms* [23, 40], while 36% express both CSF-1 and *c-fms* [23,41]. Autocrine signaling between *c-fms* and CSF-1 promotes breast cancer cell adhesion [42]. Furthermore, *c-fms* activation by CSF-1, or *c-fms* overexpression, confers an invasive, metastatic phenotype to breast cells [19,36,43,44]. Transfection of wild-type *c-fms* constructs into normal mammary epithelial cells imparts anchorage independent growth, invasiveness and metastasis [36]. In contrast, those constructs containing mutations at two major *c-fms* autophosphorylation sites show differential effects on these tumor phenotypes. Overexpression of *c-fms* in human breast cancer cells using an experimental metastasis model results in short tumor latency and extensive metastatic spread [44].

In the context of breast cancer risk, the role of circulating CSF-1 levels has been studied, with the findings dependent on menopausal status [45]. Serum levels of CSF-1 are frequently elevated in patients with metastatic breast cancer [23]; in terminally ill patients these levels reach tenfold higher than normal [11]. In breast tumors, invasive cells consistently express CSF-1 while adjacent non-invasive, *in situ* lesions do not [46], nuclear CSF-1 staining is associated with poor survival [46], and *c-fms* expression confers an increased risk for local relapse [28]. In a large breast cancer tissue array, *c-fms* is strongly associated with lymph node metastasis and poor survival [33].

CSF-1/*c-fms* in epithelial ovarian cancer initiation & metastasis

Autocrine role

Autocrine signaling between CSF-1 and *c-fms* is important for progression of epithelial ovarian cancer. The mechanisms through which *c-fms* and CSF-1 confer the invasive metastatic phenotype of several cancers, including ovarian cancers, are being investigated [9,35,36,44, 47–50]. Invasion of the extracellular matrix by epithelial ovarian cancer cells is strongly correlated with CSF-1 expression, and CSF-1-stimulated invasiveness is mediated through the actions of the urokinase plasminogen activator (uPA) [19,48], which is CSF-1 inducible [48, 50,51]. Urokinase is a serine protease involved in tissue remodeling and, like CSF-1, is elevated in many cancers. uPA has also been demonstrated to be elevated in malignant as compared with benign ovarian tumors and in comparison with normal ovarian epithelium [52,53]. uPA binding to its receptor (uPAR) has been demonstrated to result in a variety of carcinogenic processes, including invasion, adhesion, migration, proliferation and induction of signaling events that result in cellular differentiation [54], suggesting that it mediates a host of cellular activities. Furthermore, the activity of uPA appears to be dynamic, and furthermore dependent on the particular cellular environments and states it encounters [54]. Overexpression of uPA or its related PAI-1 by epithelial ovarian cancer cells within ovarian cancer specimens is predictive of a poor outcome [55,56]. In these studies, there was a significant association between tumors that expressed uPA or PAI-1, and those that expressed CSF-1. uPA expression has previously been observed to be associated with virulence of tumorigenicity of epithelial ovarian cancer cells [48]. In addition, antisense oligonucleotide-mediated inhibition of uPA expression in epithelial ovarian cancer cells decreases the extent of metastatic peritoneal spread of human ovarian cancer in nude mice [57]. Taken together, the uPA/uPAR axis appears to represent one avenue of downstream signaling for CSF-1-induced invasiveness and metastasis in ovarian cancer. Furthermore, this axis appears to be a logical therapeutic target for this disease.

To further elucidate the etiological role of CSF-1 in neoplastic progression, we recently studied whether epithelial ovarian cancer cells isolated from free-floating cells in ascites fluid of an ovarian cancer patient could be transformed, by overexpression of CSF-1, into a more invasive and metastatic counterpart [35]. Overexpressing the most abundant endogenous human CSF-1 transcript in these ascites-derived cells enables the effect of autocrine stimulation of these *c-fms*-bearing cells to be studied. This 4 kb CSF-1 transcript encodes for the secreted form of CSF-1 measured in the serum and ascites of ovarian cancer patients, which was demonstrated to have prognostic value. We first observed that this enhanced autocrine signaling led to increased invasiveness, adhesion and motility *in vitro* – all crucial factors in the metastatic cascade.

In vitro, up to 12-fold higher invasiveness through human extracellular matrix was seen with the CSF-1-overexpressing clones and two- and six-fold higher adhesion and chemoattractant-directed motility, respectively, when compared with controls. To provide evidence that this enhanced invasiveness occurring in the CSF-1-overexpressing cells was a result of the urokinase system previously described [48], a significant increase in cell-surface uPA activity by the CSF-1-overexpressing ovarian cancer cells was observed. Then, utilizing the potent synthetic uPA inhibitor B428 [58], which has been demonstrated to inhibit cell-surface uPA and surface uPA-mediated cellular degradative functions, a dose-response inhibition of invasiveness of these cells was demonstrated, compared with controls. Thus, the significant increase in cell-surface uPA activity that we demonstrated by CSF-1 appears to contribute in part to the enhanced invasiveness exhibited by the CSF-1-overexpressing cells.

In vivo, in nude mice, a virulently metastatic and invasive phenotype to ovarian cancer cells was observed by CSF-1 overexpression [35]. In these mice, intraperitoneal injection of the CSF-1-overexpressing cells produced a wide array of visceral, nodal and distant metastasis, with a degree of enhanced tumor burden not seen in any of the ten mice inoculated with transfectant control cells. Notably, organ invasion and distant metastasis was a common finding. Among the ten mice, the portal region was invaded by tumor in five cases, visceral invasion of the ovary was observed in four (visceral invasion of the liver and pancreas was less frequently observed), transdiaphragmatic invasion was noted in three, lymph node invasion in eight (most commonly affected lymph nodes were mesenteric, portal, inguinal, mediastinal, and rarely submandibular; lymph vascular space invasion was present in animals with extensive tumor infiltration) and lung metastases were observed in four mice.

In contrast to the virulence of tumors developing in 100% of mice bearing the CSF-1-overexpressing cells, complete absence of tumor-take distinguished 40% of mice implanted with transfectant control cells. Notably, disruption of this autocrine loop using antisense oligomer therapy against *c-fms* and (3'-untranslated region-mediated) knockdown of CSF-1 protein resulted in reversal of *in vitro* and *in vivo* tumor phenotypes.

Taken together, autocrine signaling by CSF-1 binding to *c-fms*, through the actions of uPA, PAI-1 and other downstream modulators, appears to play a significant role in the invasive, metastatic phenotype of epithelial ovarian cancer, thereby conferring a poor prognosis to ovarian cancer patients. Furthermore, CSF-1/*c-fms* appears to play a role in ovarian cancer tumorigenesis. This recent observation largely explains, for the first time, the poor prognosis associated with tumors that overexpress *c-fms* and *CSF-1* in their metastases [27].

Paracrine role

In addition to the autocrine roles for endogenous activation of *c-fms*-bearing, CSF-1-secreting epithelial cancer cells, CSF-1 expression by macrophages, stromal cells and osteoclasts allows for important paracrine interactions between host stroma and tumor epithelium. For instance, when lung cancer xenograft tumor growth in the mutant *op/op* mouse lacking host CSF-1 was

studied [59], poor vascularization of tumor stroma and impaired tumor growth were both observed, suggesting that downregulation of CSF-1 may be tumor inhibitory both by direct (epithelial) and indirect (stromal) means. This is clearly the case for breast cancer [43,60–62], where the role of tumor-associated macrophages bearing CSF-1 is tumor-promoting [43,63]. 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% [43,61] and improved their survival [61]. Hence, paracrine signaling by macrophages bearing CSF-1 also plays a role in breast cancer progression. Transgenic models suggest that the absence of CSF-1 has little effect on breast tumor initiation, but 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 [43].

In epithelial ovarian cancer the picture is less clear, with an apparent paradoxical protective effect of stromal *CSF-1* expression observed, although the stromal cell origin of CSF-1 was not identified [27,31]. Thus, this stromal CSF-1 staining observed in human epithelial ovarian cancer specimens may not be limited to the infiltrating tumor-associated macrophages, but likely includes expression by supporting stromal cells as well. Interestingly, in line with this observation in human ovarian cancer specimens, we recently used our *in vivo* mouse model [35] to demonstrate that those metastatic foci arising from ovarian cancer cells not expressing detectable CSF-1 (vector-transduced control cells) elicited an intense inflammatory infiltrate and were well encapsulated in a fibrous capsule. This is in striking contrast to the invasive and extensive nature of the metastatic foci arising from the CSF-1 overexpressing ovarian cancer cells, which were not associated with an inflammatory reaction [35]. Again, the infiltrating cells constituting the inflammatory reaction were not individually identified. However, others have described the *in vitro* interactions between ovarian cancer cells and macrophages to be tumor-promoting [64,65]. In addition, others have infused exogenous CSF-1 intravenously to epithelial ovarian cancer patients and have shown that this cytokine improves chemotherapy-induced impaired natural killer cell activity and granulocyte function [66], thereby decreasing the incidence of febrile neutropenia [67]. Fortunately, the effect of such infusion was observed primarily on immunological targets, with no adverse effect on patient survival noted [67]. While iatrogenic infusion of CSF-1 could be construed to result in a paracrine effect, the autocrine and paracrine interactions within the local tumor microenvironment likely predominate in determination of tumor behavior. Thus, the current data on the role of stromal CSF-1 in ovarian cancer is conflicting, although the bulk of the literature in general on tumor-associated macrophages in cancer would support a tumor-promoting role [63].

An additional observation, which is notable regarding the role of stromal CSF-1 staining in ovarian cancer [27], is that stromal CSF-1 staining is strongly associated with tumors that were low grade (either grade 1 tumors or those of low malignant potential). In fact, the presence of stromal CSF-1 is significantly correlated with a lack of strong co-expression of CSF-1 and its receptor in the tumor. This raises the possibility that co-expression of CSF-1 and its receptor in the tumor may be a marker of the high-grade molecular pathway recently elucidated, with stromal CSF-1 associated with the low-grade pathway [4].

Regulation of CSF-1 expression

In order to target the CSF-1/*c-fms* pathway, it is important to understand how the expression of CSF-1 and/or *c-fms* is regulated at a molecular level. Regulation of CSF-1 expression has been studied largely outside the context of epithelial cells, focusing on cells of monocytic lineage [68–73] or stromal cells [74–76]. During monocytic differentiation, post-transcriptional control of CSF-1 expression plays a prominent role [68–70,73], although there is a contribution at the transcriptional level [71,72]. During inhibition of monocytic differentiation by dexamethasone, no change in the CSF-1 transcription rate was observed

[68]. Instead, CSF-1 mRNA half-life was altered. During differentiation of myoblasts, *CSF-1* expression is also post-transcriptionally regulated, and associated with a change in CSF-1 mRNA half-life [74]. In contrast, in fibroblasts, the primary mode of regulation of expression of *CSF-1* is transcriptional, allowing these cells the versatility of rapid response to external stimuli, such as growth factors or serum [75].

Post-transcriptional regulation of CSF-1

There is a growing recognition of the impact of gene regulation at the post-transcriptional level on human biological processes. It is estimated that at least 50% of genes are regulated on the basis of mRNA stability by RNA-binding proteins [77]. RNA-binding proteins play an important role in the control of gene expression during early development [78], and are implicated in several human diseases [79,80]. Some RNA-binding proteins, when overexpressed, can confer tumorigenesis [81], or invasive and metastatic properties to tumors [82], while others suppress tumor growth *in vivo* [83]. Many, but not all, post-transcriptional events are regulated by the 3'-untranslated region (UTR) sequences [84]. These phenotypic changes related to 3'-UTR expression are believed to be mediated by both RNA-binding proteins, as well as the more recently recognized miRNAs. In fact, nearly half of the putative motifs in the 3'-UTR for post-transcriptional regulation are associated with miRNAs [85]. There are now reports of both oncogenic miRNAs [86,87] and miRNA tumor suppressors [88]. Circulating miRNAs are even being explored for their use in cancer detection [89]. In ovarian cancer, miR-130a has recently been described to post-transcriptionally regulate CSF-1 [90].

Previous work in epithelial ovarian cancer cells suggested that CSF-1 is regulated post-transcriptionally [91]. In epithelial ovarian cancer, by far the most abundant CSF-1 transcript is 4 kb (containing the AU-rich 3'-UTR exon 10), which encodes for the majority of secreted CSF-1 found in the serum or ascites fluid [91–93]. Alternative splicing of the CSF-1 pre-mRNA, also yields several minor CSF-1 transcripts that largely do not contain exon 10, are not AU-rich and are minimally expressed in ovarian cancer [91,93]. In contrast, the terminal 144nt of exon 10 CSF-1 is particularly AU-rich [94,95]. The AU-rich sequences in the 3'-UTR are the best-characterized of several signals that dictate mRNA decay, and more recently have also been shown to confer translational silencing [96].

Delivery of excess 3'-UTR RNA inhibits tumorigenicity, invasion and metastasis *in vivo* [97–100], potentially by titrating *trans*-acting binding factors [101]. Our recent data in ovarian cancer of the effect of excess 3'-UTR 144nt AU-rich CSF-1 serving as a 3'-UTR knockdown of CSF-1, in inhibition of tumor metastasis *in vivo*, is in line with this finding [35]. It is important to recognize that the effect of excess AU-rich 3'-UTR CSF-1 sequences is not specific to regulation of endogenous CSF-1 expression, and in sequestering other regulatory RNA-binding proteins, could impact on regulation of other targets affecting tumor phenotype.

3'UTR CSF-1 RNA-binding proteins that regulate CSF-1 expression

We sought to identify and characterize the RNA-binding proteins that recognize the 144nt AU-rich CSF-1 sequence. We have recently shown that the 144nt AU-rich region of 3'-UTR CSF-1 RNA destabilizes reporter RNA. Among several CSF-1 RNA-binding proteins that we are characterizing, we recently described GAPDH as an AU-rich RNA-binding protein that targets this 144nt CSF-1 element [47], and defined by RNA footprinting analysis, the region of the 144nt CSF-1 element to which GAPDH binds [102]. Mutations of the AU-rich regions within this footprint interfere with GAPDH binding. GAPDH promotes CSF-1 mRNA and protein levels by stabilizing CSF-1 mRNA. In ovarian cancer cells, silencing GAPDH led to a decrease in CSF-1 mRNA half-life by 50%, which is associated with a significant downregulation of

CSF-1 RNA and protein [102]. This work suggests that GAPDH, by binding the 144nt AU-rich 3'-UTR CSF-1 element, protects CSF-1 mRNA from decay, thus increasing levels of CSF-1 mRNA and protein. The work also found that GAPDH itself is regulated in ovarian cancer specimens, and that GAPDH expression is significantly associated with that of CSF-1. GAPDH has numerous functions in the nucleus and cytoplasm [103]. This report [102] is the first to identify regulation of mRNA stability as one of the many roles of the multifunctional GAPDH.

Regulation of *c-fms* expression

Both transcriptional and post-transcriptional control of *c-fms* expression play dominant roles in normal and malignant monocytes [68,104–106]. We have studied in depth the glucocorticoid regulation of *c-fms*, as it is relevant in particular to *c-fms*-related breast cancer behavior. In this context, physiological levels of glucocorticoids markedly upregulate *c-fms* expression in both breast cancer cells [23,107] and in the large majority of primary organ cultures of breast cancer specimens [108]. In an *in vivo* mouse model, antiglucocorticoids decrease endogenous *c-fms* levels in breast cancer metastases [44]. While there is an early transcriptional contribution by glucocorticoids on *c-fms* [107,109], glucocorticoid treatment for 6–24 h does not enhance *c-fms* gene transcription [110]. In choriocarcinoma cells, glucocorticoid-stimulated *c-fms* RNA and protein similarly demonstrate no detectable enhancement of *c-fms* gene transcription [25]. During inhibition of monocytic differentiation, glucocorticoids have been shown to alter *c-fms* transcript half-life [68].

3'-UTR *c-fms* RNA-binding proteins that regulate *c-fms* expression

We pursued the mechanism underlying the apparent post-transcriptional regulation of *c-fms* in epithelial cancers. The 3'-UTR of *c-fms* is not AU-rich, and the existence and identity of *c-fms* RNA regulatory proteins remained unknown until recently when we defined a novel 69nt region in the 3'-UTR of *c-fms* RNA as a binding motif for a member of the Hu family of RNA-binding proteins (HuR) [49]. HuR is ubiquitously expressed and plays a pivotal role regulating gene expression post-transcriptionally by stabilizing and/or promoting the translation of mRNAs, which contain AU-rich elements (AREs) [111]. We recently described HuR's stimulation of *c-fms* RNA and protein in breast cancer cells to be dependent on the presence of this novel RNA motif. Furthermore, we found the known glucocorticoid stimulation of *c-fms* RNA and protein in breast cancer cells to be largely dependent on HuR [49]. However, this 69nt *c-fms* sequence is not AU-rich, has no homology to known human sequences and has not been previously described to be a RNA consensus sequence for protein binding.

Overall, HuR confers a strong tumor-promoting effect in cancer by the post-transcriptional regulation of many targets [112]. In this way, the nucleocytoplasmic shuttling protein, HuR, promotes the expression of several cancer-related target mRNAs, including those of oncogenes and cytokines that have tumor-promoting roles. The non-AU-rich *c-fms* is now added to this list of HuR targets that are tumor promoting.

Owing to the stabilizing effect of HuR on cytokines and oncogenes, and now with our finding of HuR's interactions with *c-fms* RNA and its stimulation of *c-fms* expression, we and others have investigated the clinical role of HuR in cancer, including those of breast or ovarian origin [113–117]. In breast cancer, we recently found that overexpression of nuclear HuR is associated with nodal metastasis and independently with poor survival ($p = 0.03$; relative risk [RR]: 1.45), using the largest breast cancer tissue array to date to be used for this purpose ($n = 670$) [49]. Importantly, nuclear HuR was highly co-expressed with *c-fms* in the breast tumors ($p = 0.0007$). Collectively, our findings suggest that HuR promotes *c-fms*-related breast cancer progression [49].

In ovarian cancer, where *c-fms* is overexpressed in the majority of cases [27], we recently reported a study of the clinical role of HuR, showing that nuclear HuR expression is significantly associated with high grade ($p < 0.0001$) and poor disease-free survival ($p = 0.009$) [118]. HuR was expressed in the nucleus of ovarian cancer cells in 86% of cases, in line with our findings in ovarian cancer cell lines. Others have described the association of cytoplasmic HuR with high grade and poor prognosis in ovarian cancer [113,114].

Potential therapeutics targeting *c-fms*/CSF-1 signaling

The *c-fms* proto-oncogene is an ideal therapeutic target outside of pregnancy, as its expression is not detectable in normal tissues except macrophages [21,23,27,34]. There is a wider tissue distribution of CSF-1 outside of pregnancy, including macrophages, fibroblasts and osteoclasts [16]. There are an encouraging number of agents that have been developed to specifically target *c-fms* (e.g., vatalanib, ABT869, CYT645 and Ki20227), but none have yet been tested in ovarian cancer patients [15]. One US FDA-approved drug, imatinib, which targets tyrosine kinase receptor *c-kit* as well as *c-fms*, has failed to show activity in ovarian cancer [119]. Another approved multitargeted tyrosine kinase inhibitor, dasatinib, is also a potent inhibitor of tumor-associated macrophages, osteoclasts and *c-fms*. This drug is currently being tested in ovarian cancer patients.

Elucidation of downstream signaling pathways of CSF-1/*c-fms* in the regulation of cancer behavior [31,35,36,48,120] is ongoing. Therapeutic application of technologies directed against the activated uPA/uPAR axis downstream of this autocrine pathway has begun to show promise. While earlier trials in refractory ovarian cancer, which had targeted the matrix metalloproteinase pathway – a uPA-related pathway also important to invasiveness and adhesiveness of ovarian cancer cells – had not shown benefit [121], recent focus on Å6, a capped 8-amino-acid peptide derived from human single-chain uPA [122], has yielded more positive results. This peptide interferes with binding of endogenous uPA and uPAR. Encouragingly, a Phase II randomized trial of Å6 in ovarian cancer patients was associated with a statistically significant improvement in progression-free survival ($p = 0.01$), compared with patients who took placebo [123].

Elucidation of some key upstream regulators of CSF-1/*c-fms* expression has been described in this chapter. Clearly, the HuR RNA-binding protein is a logical therapeutic target for many reasons, which are not restricted to the control of cancer.

Conclusion

Improved therapeutic agents targeted to newly-validated molecular pathways that are responsible for progression of epithelial ovarian cancer are sorely needed. Approximately 75% of epithelial ovarian cancer patients present with metastatic disease throughout the peritoneal cavity, often accompanied by lymphatic spread. The CSF-1/*c-fms* pathway is now established as an important mechanism by which ovarian cancer cells impart virulent, invasive metastases. Investigation into regulatory pathways that impact on CSF-1/*c-fms* expression leads to identification of key post-transcriptional regulators that stimulate CSF-1 or *c-fms* expression. Identification of these upstream regulators adds to the list of potential downstream targets that impact on the functioning of the CSF-1/*c-fms*-signaling pathway in ovarian cancer.

Executive summary

Epithelial ovarian cancer

- While there has been recent progress in molecular understanding, there is a long way to go to effect durable treatment for the majority of patients.

- Delineating the role for colony-stimulating factor (CSF-1)/*c-fms* in the invasive, metastatic behavior of ovarian cancer, along with the molecular factors contributing to regulation of this pathway, affords additional opportunities for therapeutic targeting.

CSF-1/*c-fms* in ovarian cancer

- CSF-1/*c-fms*, as well as the activated form of *c-fms*, is observed in the majority of ovarian cancer cases, with minimal to no expression in the normal epithelium.
- CSF-1 as a tumor marker/prognostic factor:
 - At diagnosis, elevated serum and ascitic fluid CSF-1 levels predict poor outcome.
 - Serum CSF-1 can be used as a tumor marker heralding progression of the disease, and as part of a panel of markers may help detect ovarian cancer.
- An autocrine role for binding of CSF-1 to *c-fms* has been established in this disease.
 - Co-expression of CSF-1 and *c-fms* in metastases is an independent poor prognostic factor; none of the tumors of low malignant potential, which by definition are noninvasive, express both cytokine and receptor.
 - Overexpression of endogenous CSF-1 transforms *c-fms*-bearing ovarian cancer cells free-floating in ascites fluid to those that are significantly more invasive, motile and adhesive *in vitro*, and more tumorigenic and invasively metastatic *in vivo*.
 - Inhibition of this CSF-1/*c-fms* pathway decreases both invasiveness *in vitro* and metastatic spread *in vivo*.
 - One downstream effector of CSF-1/*c-fms* signaling impacting on ovarian cancer behavior is the urokinase plasminogen activator (uPA)/uPA receptor axis.
- A paracrine role for other sources of CSF-1 impacting on ovarian cancer behavior is conflicting to date.

Post-transcriptional regulation of CSF-1 in ovarian cancer

- The 3'-untranslated region (3'-UTR) adenylate uridylylate-rich element of CSF-1 RNA is defined as a destabilizing element.
- GAPDH specifically binds this element and stimulates CSF-1 expression.
- GAPDH itself is regulated in ovarian cancers and is associated with CSF-1.

Post-transcriptional regulation of *c-fms*

- A novel 3'-UTR non-AU-rich element in *c-fms* has been defined that promotes *c-fms* expression.
 - HuR binds to this element, regulates *c-fms* post-transcriptionally and is largely responsible for glucocorticoid stimulation of *c-fms* expression in breast cancer cells.
 - Nuclear HuR is a poor prognostic factor in both breast and ovarian cancer.

Future perspective

We are in a position now, having sufficient evidence that supports the autocrine role of CSF-1/*c-fms* signaling in neoplastic progression of epithelial ovarian cancer, to test therapeutics that target this pathway directly. Approaches could involve both RNA and/or protein targets. These targets are likely to include some of those recently identified upstream regulators of CSF-1/*c-fms* expression, CSF-1 and/or *c-fms*, or those activated downstream factors resulting from binding between CSF-1 and *c-fms*. In the future, there will be a better understanding of the role of the CSF-1-expressing tumor-associated macrophage in ovarian cancer behavior. If it becomes clear that paracrine, in addition to the recognized autocrine, sources of CSF-1 are an appropriate target for this disease, then this dual approach will significantly enhance the chances for therapeutic success. Based on the therapeutic experience in ovarian cancer to date, ultimately, it is likely that combinations targeting more than one key signaling pathway will be needed to overcome the inherent redundancy and multiple gene interactions that govern ovarian cancer pathogenesis and progression.

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Figure 1. CSF-1-stimulated activation of cancer cells parallels that of differentiation and activation of macrophages and osteoclasts

The metastatic cancer cells, macrophages, osteoclasts and their precursors all express *c-fms* and are responsive to CSF-1.

CFU-M: Colony forming unit-macrophage; CSF-1: Macrophage colony-stimulating factor.