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ORIGINAL PAPERS

Microenvironmental Influences on Metastasis Suppressor Expression and Function during a Metastatic Cell's Journey

Wen Liu · Carolyn J. Vivian · Amanda E. Brinker · Kelsey R. Hampton · Evi Lianidou · Danny R. Welch

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Abstract Metastasis is the process of primary tumor cells breaking away and colonizing distant secondary sites. In order for a tumor cell growing in one microenvironment to travel to, and flourish in, a secondary environment, it must survive a series of events termed the metastatic cascade. Before departing the primary tumor, cells acquire genetic and epigenetic changes that endow them with properties not usually associated with related normal differentiated cells. Those cells also induce a subset of bone marrow-derived stem cells to mobilize and establish pre-metastatic niches [1]. Many tumor cells undergo epithelial-to-mesenchymal transition (EMT), where they transiently acquire morphologic changes, reduced requirements for cell-cell contact and become more invasive [2]. Invasive tumor cells eventually enter the circulatory (hematogenous) or lymphatic systems or travel across body cavities. In transit, tumor cells must resist anoikis, survive sheer forces and evade detection by the immune system. For blood-borne metastases, surviving cells then arrest or adhere to endothelial linings before either proliferating or extravasating. Eventually, tumor cells complete the process by proliferating to form a macroscopic mass [3].

Up to 90 % of all cancer related morbidity and mortality can be attributed to metastasis. Surgery manages to ablate most primary tumors, especially when combined with chemotherapy and radiation. But if cells have disseminated, survival rates drop precipitously. While multiple parameters of the primary tumor are predictive of local or distant relapse, biopsies remain an imperfect science. The introduction of molecular and other biomarkers [4, 5] continue to improve the accuracy of prognosis. However, the invasive procedure introduces new complications for the patient. Likewise, the heterogeneity of any tumor population [3, 6, 7] means that sampling error (i.e., since it is impractical to examine the entire tumor) necessitates further improvements.

In the case of breast cancer, for example, women diagnosed with stage I diseases (i.e., no evidence of invasion through a basement membrane) still have a ~30 % likelihood of developing distant metastases [8]. Many physicians and patients opt for additional chemotherapy in order to "mop up" cells that have disseminated and have the potential to grow into macroscopic metastases. This means that~70 % of patients receive unnecessary therapy, which has undesirable side effects. Therefore, improving prognostic capability is highly desirable.

Recent advances allow profiling of primary tumor DNA sequences and gene expression patterns to define a so-called metastatic signature [9–11], which can be predictive of patient outcome. However, the genetic changes that a tumor cell must undergo to survive the initial events of the metastatic cascade and colonize a second location belie a plasticity that may not be adequately captured in a sampling of heterogeneous tumors. In order to tailor or personalize patient treatments, a more accurate assessment of the genetic profile in the metastases is needed. Biopsy of each individual metastasis is not practical, safe, nor particularly cost-effective. In recent years, there has been a resurrection of the notion to do a 'liquid biopsy,' which essentially involves sampling of circulating tumor cells (CTC) and/or cell free nucleic acids (cfDNA, including microRNA (miRNA)) present in blood and lymph [12–16].

W. Liu · C. J. Vivian · A. E. Brinker · K. R. Hampton · D. R. Welch (✉)
Department of Cancer Biology, University of Kansas Cancer Center, Kansas City, KS 66160, USA
e-mail: DWelch@KUMC.edu

E. Lianidou
Lab of Analytical Chemistry, Department of Chemistry, University of Athens, Athens 15771, GREECE

D. R. Welch
University of Kansas Cancer Center, University of Kansas Medical Center, Kansas City 66160, KS, USA

The rationale for liquid biopsy is that tumors shed cells and/or genetic fragments into the circulation, theoretically making the blood representative of not only the primary tumor but also distant metastases. Logically, one would predict that the proportion of CTC and/or cfDNA would be proportionate to the likelihood of developing metastases [14]. While a linear relationship does not exist, the information within CTC or cfDNA is beginning to show great promise for enabling a global snapshot of the disease. However, the CTC and cfDNA are present at extremely low levels. Nonetheless, newer technologies capture enough material to enrich and sequence the patient's DNA or quantification of some biomarkers.

Among the biomarkers showing great promise are metastasis suppressors which, by definition, block a tumor cell's ability to complete the metastatic process without prohibiting primary tumor growth [17]. Since the discovery of the first metastasis suppressor, Nm23, more than 30 have been functionally characterized. They function at various stages of the metastatic cascade, but their mechanisms of action, for the most part, remain ill-defined. Deciphering the molecular interactions of functional metastasis suppressors may provide insights for targeted therapies when these regulators cease to function and result in metastatic disease.

In this brief review, we summarize what is known about the various metastasis suppressors and their functions at individual steps of the metastatic cascade (Table 1). Some of the subdivisions are rather arbitrary in nature, since many metastasis suppressors affect more than one step in the metastatic cascade. Nonetheless what emerges is a realization that metastasis suppressors are intimately associated with the microenvironments in which cancer cells find themselves [18].

Keywords BRMS1 · CD44 · CRMP4 · DCC · DLC1 · GSN · LIFR · LSD1 · MTBP · OGR1 · RKIP · SSeCKS · Stefin A · RhoGDI2 · RRM1 · Caspase 8 · Gas1. KAI1 · Regulatory RNA · miRNA · KISS1 · NDRG1 · NME1 · MKK4 · MKK7 · p38 · CADM1 · TSLC1 · FXR · Invasion · Motility · Metastasis suppressor · Colonization · Cell-free DNA · Circulating tumor cell · CTC · DTC · cfDNA

Metastasis Suppressors That Regulate Growth, Angiogenesis, Local Invasion and/or EMT

BRMS1

The Breast cancer metastasis suppressor-1 (BRMS1) gene, which was discovered by differential display comparing metastasis-competent and metastasis-suppressed cells [19], encodes a predominantly nuclear protein, which interacts with several nuclear proteins associated with large SIN3:HDAC (histone deacetylase) chromatin remodeling complexes. As a result, BRMS1 is thought to control metastasis by regulating multiple genes which are intimately involved in the metastatic cascade, such as genes that control apoptosis, cell-cell communication, and cell migration [20]. However, this proposed mechanism of action is probably simplistic since cellular location is critical [21–25]. A recent paper suggests that BRMS1 suppresses lung cancer metastasis through an E3 ligase function when associated with histone acetyltransferase p300 [26]; so, cytoplasmic functions or modifications of non-histone proteins may be involved. The latter functions have still not been fully defined. Other data suggest that BRMS1 may be a critical player in cellular communication with the microenvironment, controlling phosphoinositide [27, 28] and sphingosine kinase signaling [29]. From clinical data, BRMS1 localization and metastasis appear to be cell-type dependent [21, 23]. Therefore, the mechanisms of action for BRMS1 metastasis suppression may vary, depending upon the cell of origin.

Most data show that the initial steps of the metastatic cascade (e.g., local invasion and intravasation) are individually modestly affected by BRMS1 expression [30–32]. BRMS1 decreases survival of CTC by increasing sensitivity to anoikis [33], and the few cells that successfully seed other organs are less capable of colonizing them [33]. A recent study addressed how BRMS1 expression in CTC affects that cell's ability to respond to microenvironment, to reorganize cytoskeleton and form cell-matrix interactions [25]. Taken together, these data show that BRMS1 functions at the interface between various microenvironments and tumor cell behavior. As with other metastasis suppressors, few mutations have been found in the coding regions of the genes. However, differential expression appears to be more critical with regard to their ability to successfully suppress metastasis. This observation has led to exploration of the promoter methylation patterns for the metastasis suppressors. The BRMS1 promoter contains several CpG islands which can become methylated and effect gene expression [34]. Chimonidou et al. recently assessed CTC from breast cancer patients for BRMS1 expression and BRMS1 promoter methylation and found that reduced expression and promoter methylation correspond to probability of recurrence from metastasis and survival [35]. Similar correlations were found in non-small cell lung cancer cfDNA [36].

CD44

CD44 is membrane adhesion molecule that mediates epithelial-stromal and epithelial-matrix interactions that can direct organization of ECM and intracellular signaling. Binding to a variety of actin-cytoskeleton adaptor proteins and signaling mediators enables CD44 to also direct cytoskeletal organization and mediate cell adhesion and motility [37–39]. A definitive role for CD44 in metastasis suppression is more inferred than directly demonstrated, given its role as a

biomarker for cancer cell 'stem-ness' [40]. Nonetheless, there is suggestive evidence that CD44 can function as a metastasis suppressor. Its expression is inversely correlated with metastatic potential in prostate cancer cell lines and $CD44^{-/-}$ matings with MMTV-PyMT mice have increased lung metastasis without changing primary tumor growth [41].

The mechanism of action for CD44 is amazingly complex owing to its post-translational cleavage at both the extracellular and cytoplasmic domains, its interactions with numerous cancer-associated factors, and its involvement in cell-cell and cell-matrix adhesion, cell signaling, survival, growth, 'stem-ness' and migration. Depending on the extracellular factor and/or intracellular signaling with which CD44 is engaged, it plays dual roles in promoting and suppressing tumor progression and/or metastasis [40, 42]. For these reasons, CD44 highlights the importance of understanding post-transcriptional and -translational modifications as well as defining interactions before ascribing specific functions to a metastasis suppressor.

Collapsin Response Mediator Protein 4 (CRMP4)

CRMP4 was identified in prostate cancer by 2D-DIGE (two-dimensional differential gel electrophoresis) proteomic analysis [43]. Expression of both CRMP4 mRNA and protein is inversely associated with lymph node metastasis of prostate cancer. Overexpression in prostate cancer cells leads to lowered invasion in vitro as well as fewer metastases in vivo. CRMP4 belongs to a large family of collapsin proteins which regulate axon guidance and neurite outgrowth [44, 45]. Since many of the functions involved in these processes parallel those that take place in metastasis, one can hypothesize that CRMP4 is regulating chemotactic responses, motility and invasion. Since other members of the collapsin family have been associated with regulating tumor invasion [46], the hypothesis is consistent with available data. However, CRMP1 has not yet been shown to affect metastasis in vivo. Another mechanism of action was reported recently – CRMP4 is a physiological substrate of GSK3 during mitotic chromosomal alignment by promoting cytoskeletal remodeling [47]. Whether this action of CRMP4 is relevant in metastasis suppression remains to be elucidated.

Deleted in Colorectal Cancer (DCC)

DCC encodes the receptor for the axon guidance molecule netrin and, like CRMP4, functions during neural development to control survival and migration. Like BRMS1, DCC and netrin affect multiple steps in the metastatic cascade. Because DCC induces apoptosis in the absence of netrin, it is a selective advantage for tumor cells to lose DCC expression or gain netrin expression. Loss of DCC promotes metastasis formation without affecting the primary tumor latency in a mouse model of mammary carcinoma [48]. On the other hand, netrin expression favors survival and angiogenesis of metastatic tumor cells by inhibiting the pro-apoptotic effects [49]. Interestingly, netrin-DCC signaling regulates a morphologic change of epithelial cells during *Drosophila* wing eversion [50], which is highly reminiscent of EMT.

Down-regulation of DCC in breast cancer cells has been generally associated with worse prognosis and higher risk of recurrent disease. Loss of DCC gene expression has been correlated with more advanced stage of ovarian and gastric cancer [51, 52]. Diminished DCC protein expression via loss of heterozygosity for the *DCC* gene has also contributes to colorectal and pancreatic tumor dissemination [53], while up-regulation of netrin provides a growth advantage to various cancers including breast, neuroblastoma, pancreatic and lung as well as providing protection from hypoxia-induced apoptosis in mesenchymal stem cells expressing DCC [54, 55]. Together, these observations raised the possibility that netrin-DCC signaling can modulate metastasis by interfering with signals from the microenvironment.

Deleted in Liver Cancer 1 (DLC1)

DLC1 was identified as a breast cancer metastasis suppressor using microarray-based transcriptional profiling of cell lines with different metastatic efficiency [56]. Re-expression of DLC-1 in metastatic breast cancer cells inhibits migration and invasion in vitro and lung metastasis in vivo, but does not alter tumorigenicity. Down-regulation of DLC-1 is negatively associated with tumor progression in breast cancer, clear cell renal and urothelial carcinomas [57–59]. DLC1 serves as a GTPase activating protein (GAP) particularly for RhoA, B, and C but also Cdc42, affecting cell polarity, actin organization and proliferation [60]. Very little is known about the mechanism of DLC1 in metastasis, but it seems to play specific roles in different aspects of Rho GTPase function. Over-expression leads to changes in cytoskeletal structure, focal adhesions and cellular protrusions. DLC1 activation may also play a role in sensing extracellular factor-induced stress at secondary sites. DLC1 is often inactivated due to genomic deletion or promoter hypermethylation [57].

Gelsolin (GSN)

Gelsolin is an actin-regulatory protein. Expression is often decreased in tumor cells [61]. Its role as a metastasis suppressor was first described in B16-BL6 melanoma cells [61]. Gelsolin suppressed motility in vitro and lung metastasis in vivo, while truncation of its carboxy-terminus abolished such effects. Ascribing mechanism of action is complex since there are apparently conflicting observations. A recent report from Yuan et al. revealed the crucial role of transcription factor ATF3 to suppress metastasis of bladder cancer cells via up-regulation of

gelsolin-mediated actin remodeling [62]. However, Marino et al. described that gelsolin over-expression increased metastasis, an effect which could be ablated by co-expression of the metastasis suppressor Nm23-H1 [63]. Still other studies suggest that gelsolin-expressing cells are more sensitive to apoptotic cell death [61]. It is unclear the cellular context in which gelsolin-involved apoptosis occurs.

Leukemia Inhibitory Factor Receptor (LIFR)

LIFR was first identified as tumor suppressor in breast cancer using RNA interference-based screening [64]. However, LIFR was also defined as a breast cancer metastasis suppressor subsequently [65]. LIFR acts as a downstream target of miR9, which was previously implicated as a metastasis promoter in E-cadherin-negative breast cancer cells. Re-expressing LIFR diminished local invasion and metastasis in vivo, presumably by inhibiting extravasation and colonization. It is not clear whether LIFR1-suppression of primary tumor and metastasis growth are due to distinct functions.

Opposing activities of the transcriptional co-activator YAP and TAZ (downstream effectors of Hippo signaling) appear to be essential for LIFR-mediated metastasis suppression [65]. A recent study from Hynes laboratory [66] further indicates a role for YAP in breast cancer metastasis. Thus, it will be interesting to determine whether TAZ plays a role in breast cancer metastasis. Because only a subset of primary tumors which lose LIFR form metastases, it will be interesting to determine whether loss of LIFR expression is always associated with YAP and/or TAZ activation in the same cells.

Lysine-Specific Demethylase 1 (LSD1)

LSD1 is an integral component of several co-repressor complexes including CoREST, CtBP, HDAC1/2 and Mi-2/nucleosome remodeling and deacetylase (NuRD) complex, which remove the methylation of H3K4 [67]. LSD1 functions as a metastasis suppressor in breast cancer models through modulation of TGFβ1 and EMT [68]. In contrast, LSD1 has been linked with high-risk tumors and evidence suggests that LSD1 expression leads to tumorigenesis and poor clinical outcomes in prostate, colon and ovarian cancers, as well as esophageal squamous cell carcinoma [69–71]. Pertinent to its metastasis suppression capability, LSD1 interacts with Snail1, a key regulator of EMT [68]. The downstream transcriptional effectors of LSD1 that mediate metastasis control remain to be identified.

MDM2 Binding Protein (MTBP)

MTBP first was identified as an MDM2 interacting partner during elucidation of the mechanisms of MDM2 in tumorigenesis via p53-independent mechanisms. Independent studies showed that MTBP appears to function as a metastasis suppressor [72–74]. Mtbp/p53 double heterozygous mice developed a significantly higher rate of metastatic tumors without any difference in tumor onset. Overexpression of MTBP significantly reduced metastasis formation of highly metastatic human osteosarcoma cells in animal models with little effect on primary tumor growth. MTBP inhibits F-actin crosslinking, leading to decreased filopodia formation which, in turn, reduces migration in cells lacking both MDM2 and p53. The latter interaction appears to rely on interactions with α-actinin-4 (ACTN4) [72]. It is worth mentioning that nuclear MTBP is not necessary for suppression of cell migration, which raises the question of other MTBP functions that might be responsible for suppression of metastasis.

Ovarian Cancer G-Protein Coupled Receptor 1 (OGR1)

OGR1, originally cloned from human ovarian cancer, also suppresses melanoma metastasis [75]. It is also among the most down-regulated genes in metastatic prostate cancer as well. OGR1 inhibits cell migration and transendothelial migration via increased expression of $G_{\alpha i1}$ and inhibitory secretion factors. The differential effect of OGR1 on primary tumor growth and metastasis is tumor-type dependent and may be related to its proton-sensing activity [76, 77], which is mediated by a $G_{\alpha q,}$, but this conclusion has not been confirmed by independent experimental data. Intriguingly, OGR1 deficient mice have decreased peritoneal M2, but not M1, polarized macrophages [78], which implicates immune cell infiltration and/or functionality in the anti-metastatic actions. And finally, recent reports indicate that OGR1 stimulates prostaglandin E2 (PGE2) expression of human osteoblast-like cells in response to acidic extracellular environments [79]. Connecting these apparently random observations are the well-established cross-talk between tumor cells and osteoblasts [80–82], responsiveness of tumor cells to PGE2 signaling [83] and emerging data that PGE2 can control immune cell differentiation states [83–85]. Thus, OGR1 appears to be a critical mediator of tumor cell microenvironmental interactions.

Raf Kinase Inhibitor Protein (RKIP)

RKIP was initially identified as a phospholipid binding protein, which was later shown to interact and inhibit Raf1-mediated activation of MAPK signaling. RKIP inhibits invasion and acts as a metastasis suppressor in prostate cancer [86]. RKIP expression is decreased in a number of different cancers and it is postulated that its effects on signaling are key to activity. Rosner and colleagues showed that RKIP inhibits breast cancer metastasis, in part, via suppression of let-7 and downstream genes, HMGA2 and BACH1. Together, those downstream mediators regulate invasion, homing and osteolysis [87–90]. Das et al. showed that RKIP interferes

with melanoma differentiation associated gene-9 (MDA-9)-mediated focal formation followed by suppression of melanoma metastasis [91]. RKIP also de-represses GSK3β inhibition which, in turn, inhibits β-catenin, Snail and Slug activation with expected modulation of EMT [92]. Recent data, also from the Rosner group, show that RKIP induces and HMGA2 inhibits miR-200b expression which, in turn, inhibits expression of lysyl oxidase (LOX) [93]. Also via down-regulation of HMGA2, RKIP inhibits syndecan-2 independently of miR-200 [93].

Since RKIP can also enhance sensitivity to some chemotherapeutic agents and other stresses [94], it has also been suggested that RKIP loss could be a mechanism exploited to avoid immune surveillance. Thus, the notion of interplay with the microenvironment is established. Since several cancer-related and metastasis-related upstream factors regulate RKIP (e.g., Snail, miR-224, and EZH2), environmental cues are again implicated [92, 95, 96].

Src-Suppressed Protein Kinase C Substrate (SSeCKS)

SSeCKS is a scaffolding protein that controls mitogenic signaling and cytoskeletal remodeling by binding key signaling mediators. Following crosslinking to the actin cytoskeleton and plasma membrane, SSeCKs translocates and correspondingly transports macromolecules such as Src, PKC, PKA, F-actin, calmodulin, cyclins and phospholipids to other organelles. SSeCKS re-expression decreases invasion and invadosome formation by disengaging Src from activating downstream RhoGTPase and/or PKC-Raf/MEK/ERK-mediated pathways which, in turn, control MMP expression [97–99].

Dependent on cell type, SSeCKS affects multiple different steps of tumor metastasis. Gelman and colleagues have shown that SSeCKS re-expression in rat prostate cancer cells reduces metastasis by affecting neovascularization at distal sites, but not by inhibiting tumor cell motility or proliferation [97, 100, 101]. Using a spontaneous metastasis model, Akakura et al. showed that B16F10 melanoma cells developed significantly more metastases in SSeCKS-null mice with no difference on primary tumor growth [102]. Thus, SSeCKS performs its metastasis suppressor activity using both tumor cell-autonomous and non cell-autonomous mechanisms.

Stefin A

Stefin A suppresses metastasis in human esophageal squamous cell carcinoma and murine mammary carcinomas [103, 104]. It is an endogenous inhibitor of cathepsin B, a cysteine protease, which promotes invasion [105]. Alterations in the balance between cathepsin B and stefin A (which is also true for other protease inhibitor interactions, such as matrix metalloproteinases and TIMPs) regulate invasiveness (and metastasis) in a variety of malignant tumors. Stefin A expression correlated with disease-free survival and less distant metastasis in breast cancer patients [103].

Interestingly, Stefin A-positive metastatic nodules are significantly larger than Stefin A negative ones. While this observation is described at metastatic sites in vivo, it has not yet been replicated in cell culture conditions, suggesting that expression is environmentally regulated. These observations are reminiscent of EMT and a corresponding mesenchymal-to-epithelial reversal proposed at sites of metastasis [106, 107]. By analogy, loss of Stefin A expression at primary sites may allow tumor cell dissemination, but reactivation at distant sites could inhibit outgrowth.

RhoGDI2

RhoGDI2 was originally implicated in bladder cancer metastasis suppression, but is also involved in other cancer types [108]. RhoGDI2 belongs to a small family of proteins acting as RhoGTPase inhibitors by reducing dissociation of GDP from Rho proteins. Src interacts with and phosphorylates RhoGDI2 to enhance the metastasis suppressive effects [109]. Recently, Said et al. showed that RhoGDI2-deficient disseminated tumor cells secrete several soluble factors (i.e., ECM molecules, versican, CCL2 and IL6) that stimulate macrophage recruitment [110–112]. The resulting 'inflammation storm' induces formation of a pulmonary pre-metastatic niche. Modulation of microenvironment is necessary for the RhoGDI2-induced metastasis suppression because targeting inflammation-inducing factors/signaling phenocopies the metastasis suppressor effect of RhoGDI2. Consistently, the importance of RhoGDI2 in modulation of the tumor microenvironment could be extended from previous study, which showed that loss of RhoGDI2 expression induced activation of Endothelin1 followed by enhanced migration, invasion and macrophage infiltration [113, 114].

Ribonucleotide Reductase Subunit M1 (RRM1)

RRM1 was confirmed as the molecule responsible for acquisition of metastatic potential that occurs with loss of heterozygosity on chromosome 11p15.5 in multiple human tumors [115, 116]. RRM1 encodes the regulatory subunit of ribonucleotide reductase, the heterodimeric enzyme that catalyzes the rate-limiting step in deoxyribonucleotide synthesis. Ribonucleotide reductase has central functions in DNA synthesis, growth, tumor metastasis, and drug resistance of cancer cells, and therefore is considered as an attractive target for anticancer agents.

Over-expression of RRM1 reduced experimental and spontaneous lung metastasis [115, 117]. The link between the enzymatic activity of RRM1 and its function to suppress metastasis is unclear. However, RRM1 induced PTEN

expression, which seems necessary for RRM1 decreased migration, invasion and metastasis formation in vivo [115]. Clinical studies show that RRM1 and PTEN are prognostic markers for disease-free and overall survival in NSCLC, bladder, and pancreatic cancer. Interestingly, RRM1 expression is presumably associated with gemcitabine resistance in patients with advanced lung cancer because Gemcitabine can directly bind and inactivate RRM1 [118].

Metastasis Suppressors That Affect Cell Transit and Adhesion

Caspase 8

Caspases are cysteine proteases known for their role in the receptor-mediated intrinsic pathway of apoptosis. Caspase 8 (MACH/FLICE/Mch5) contains a FADD (Fas-associating protein with death domain) homology domain and is the first enzyme of the proteolytic cascade activated by the Fas ligand (FasL), and by tumor necrosis factor (TNF) [119]. The FasL recognizes and binds to Fas (Apo-1/CD95) receptor which then can induce apoptosis in FasL sensitive cells. Suppression of death receptor induced apoptosis may play a role in the pathogenesis of some tumors. [120]. Death receptors, such as Fas, lead to formation of the death inducing signaling complex (DISC) which recruits and activates caspase 8 [121].

Caspase 8 is also linked to integrin-mediated regulation of apoptosis. Signals important for survival, migration, growth and differentiation are transduced across the cell membrane via integrin heterodimers. Integrin expression and signaling are regulated in response to growth factors and are important for tumor establishment [122] and survival. Loss of integrin-mediated adhesion is a driving signal for a cell to undergo anoikis [122]. Even if cells are adherent, but are attached using the 'wrong' integrin, they can be induced to undergo apoptosis [123–126].

In neuroblastoma, metastasis was observed in tumors where caspase 8 was deficient, whereas metastasis appeared less frequently in tumors expressing caspase 8 [127], indicating its role as a metastasis suppressor. Caspase 8 appears to determine cell survival during metastasis, but not necessarily growth of the primary tumor. Somehow, loss of caspase 8 provides a survival advantage to the metastatic cell. Another important mechanism is integrin-mediated death in which caspase 8 is recruited to and activated by clusters of unligated integrins to the cell surface independent of FADD [126]. This is important because integrin-mediated adhesion promotes cell survival, but could play an important role in determining the tropism of metastasis [124]. The roles of caspase 8 in metastasis highlight the continuous interplay between tumor cells and the environment. Even when redundant mechanisms exist (i.e., adherence to non-preferred ligands), they may not be as efficient nor sufficient to overcome the biochemical signals impinging upon the tumor cell.

Growth Arrest Specific 1 (Gas1)

Gas1 was first identified as a cell cycle arrest gene when its expression was down-regulated after growth induction of arrested NIH3T3 cells [128]. *Gas1* encodes a GPI-anchored membrane protein [129] that is a cell cycle inhibitor (G_0 to S phase transition) since Gas1 over-expressing cells do not incorporate BrdU. Gas1 was first identified as a metastatic suppressor in a genome wide shRNA screen in B16-F10 melanoma cells [130]. Gas1 plays a role in several tumors, including colorectal, prostate, bladder, and melanoma. Gas1 regulates apoptosis [131] since Gas1 suppression renders some tumor cells resistant to drug-induced apoptosis.

Gas1 over-expression also significantly reduces tumorigenicity in glioma, lung and gastric cancers. The Gas1 protein associates with Sonic Hedgehog (SHH) and Indian Hedgehog (IHH), secreted proteins that can signal to adjacent or distant cells. SHH transcription is linked to specific types of tumors. Likewise, Gas1 can be induced by Wnt proteins which encode proteins that bind to SHH [132]. Gas1 appears to suppress metastasis by regulating/inducing apoptosis through caspases 3 and 9 after disseminated tumor cells arrive at metastatic sites.

KAI1/CD82 (Kang-ai 1)

KAI1, located on human chromosome 11p11.2, and was first identified as a metastasis suppressor from a microcell-mediated chromosome transfer (MMCT) of chromosome 11 into highly metastatic rat prostate cancer cells [133]. Subsequently, its role as a metastasis suppressor has been buttressed with data in diverse cell types. KAI1 belongs to a large family of membrane glycoproteins, including ME491/ME491/CD63, MRP-1, TAPA-l, CD37, and CD53. However, not all tetraspanin family members are metastasis suppressors.

Metastasis suppression is presumably due to inhibition of cell motility/invasion, induction of senescence or induction of apoptosis. KAI1 impedes dissemination via crosstalk between KAI1/CD82 on the metastasizing tumor cell and Duffy antigen receptor for cytokines (DARC) on the adjacent vascular cells [134]. When KAI1 expressing tumor cells and DARC expressing endothelial cells interact, tumor cell proliferation is inhibited and senescence is induced [134]. KAI1 can also increase apoptosis and senescence after tumor cells have migrated to secondary sites by decreasing intracellular β-catenin/Wnt pools and through packaging and secretion in exosomes [135].

1.1. Non-Coding RNA and Regulatory RNA

Non-coding RNA (ncRNA) make up a diverse group of RNA molecules, including ribosomal (rRNA), transfer (tRNA), micro (miRNA), long non-coding (lncRNA), small nucleolar (snoRNA), small interfering (siRNA), small nuclear (snRNA) and piwi-interacting (piRNA) RNA [136]. ncRNA and miRNA can repress protein expression by means of inhibition of translation and promoting degradation. miRNA are involved in post-transcriptional modification as well as negative regulation of gene expression. When acting as effectors of tumor formation, they are termed *oncomir*. Similarly, metastasis regulatory miRNA are termed *metastamir* [137]. Due to space limitations, readers are referred to recent excellent reviews showing metastamir involvement in metastasis suppression [4, 137–140]. However, the roles of other regulatory RNA in metastasis are just beginning to be identified. For example, HOTAIR (Hox antisense intergenic RNA) was the first lncRNA associated with metastasis. HOTAIR interacts with PRC2 complex which in turn transcriptionally silences metastasis suppressor genes [141, 142]. Interestingly, miRNA and lncRNA pathways can interact to create complex regulatory networks [143], but systematic evaluation of roles in metastasis have not yet been established [144]. To our knowledge, the other regulatory RNA have not yet been described as regulators of metastasis, but we feel certain that this situation will change in the near future.

Metastasis Suppressors Affecting Colonization

KISS1

Discovered to suppress metastasis in malignant melanoma via subtractive hybridization, KISS1 is a metastasis suppressor protein mapping to chromosome 1q32 [145, 146]. KISS1 is a proprotein requiring secretion and proteolytic processing by furin outside of the cell to suppress metastasis [147]. The peptide products of KISS1 are called kisspeptins (KP). The known bioactive fragment of KISS1 (KP54) is a 54 amino acid peptide that binds a G-protein receptor, KISS1R (formerly GPR54 or AXOR12 of the $G_{q/11}$ class). KISS1R activation activates MAPK signaling, mobilizes intracellular Ca^{2+}, arrest of cell cycle, and down-regulates MMP9 through inhibition of NFkB signaling [148–150]. KISS1 and KISS1R also play important roles in the regulation of puberty through the hypothalamus-pituitary-gonadal axis [151].

Cells expressing KISS1 are able to accomplish every step of the metastatic cascade except colonization of secondary sites. Clinical reports generally correlate KISS1 expression with more favorable prognosis, except in cases of hepatocellular carcinoma [145]. The exact mechanism of KISS1-mediated metastasis suppression remains unclear, as KISS1 retains the ability to suppress metastasis even in the absence of KISS1R on the tumor cells. This observation leads to the hypothesis that paracrine mechanisms may be involved, but evidence to that mechanism is lacking. Recent reports suggest an intracellular interaction between KISS1 and PGC1α leading to increased mitochondrial biogenesis and a reversal of the Warburg Effect [152]. At this time, it can only be speculated how KISS1 regulation of metabolism could control colonization.

N-myc Downstream Regulated Gene 1 (NDRG1)

NDRG1 was identified as a metastasis suppressor gene in colon cancer. Loss of NDRG1 expression is generally correlated with an increase in cancer invasion and metastasis [153]. NDRG1 is associated with the cycling of E-cadherin to the cell surface, suggesting a role in reversing the invasive phenotype upon colonization of a new tissue [154]. There is also evidence that NDRG1 interacts with LRP6, a Wnt co-receptor to block Wnt signaling, inhibiting activation of β-catenin, rendering a non-proliferative phenotype [154]. NDRG1 also has the capacity to block the Ras/Raf and NFkB pathways, attenuating proliferation and down-regulating invasive phenotypes [153]. NDRG1 has also been implicated in blocking cellular motility by inhibiting g-actin-ARP2/3 polymerization to generate stress fibers, and the inhibition of ROCK to block formation of myosin light chain complexes for contractile motion [153].

NM23-H1 (NME1)

NM23 (non-metastatic clone 23) was discovered by Patricia Steeg by differential colony hybridization of K1735 melanoma cell clones [155]. Reports of NM23 as a metastasis suppressor have been muddled because of imprecision regarding which isoform was being tested. In general, NM23 decreases the efficiency of events leading to metastasis without altering primary tumor proliferation by the regulation of cell differentiation marker proteins [156]. With the ability to have multiple different functions, NM23 plays roles in several signaling pathways. NM23 can block MAPK signaling, prevent actin polymerization which, in turn, blocks cell motility and stops proliferation at secondary tissues [157]. Acting as a transcription factor regulator, NM23 can also down-regulate growth factors (e.g. Wnt5b), matrix metalloproteinase proteins, and apoptosis inhibitor proteins [158]. These data have been found in multiple cell lines, however, so a universal effect is not thoroughly understood. Clinically, an inverse correlation has been seen with NM23 in breast cancer, melanoma, ovarian cancer, and hepatocellular carcinoma [156]. While NM23 can down-regulate proliferation and migratory genes in a secondary tissue, it is a versatile protein which has been shown to suppress metastasis at multiple steps in the metastatic cascade.

Table 1 Metastasis suppressors and their proposed mechanisms of action

Metastasis Suppressor	Aliases	Proposed Function(s) [2]	Step(s) in metastasis inhibited [3]
BRMS1		SIN3-HDAC complexes (Chromatin modeling) Reduce phosphoinositide signaling Restore gap junction communication	Invasion Transport Colonization
CADM1	TSLC1 IgSF4 Necl2 Syncam	Up-regulates caspase-3, BAX and p21 Cytoskeletal remodeling Down-regulate MMP Cell cycle arrest Apoptosis Invasion	Colonization
Caspase-8		Induce apoptosis/anoikis Cell cycle arrest Integrin mediated death (IMD)	Survival Transport Invasion Colonization
CD44	CDW44 CSPG8 ECMR-III	Binds to hyaluronic acid receptor Cell-cell and cell-matrix adhesion	Migration
CRMP4	DRP-3 ULIP-1	Cytoskeletal remodeling	Invasion
DCC	CRC18 CRCR1 MRMV1	Cytoskeletal remodeling Regulate MAPK signaling Cell cycle arrest Promote apoptosis	Transport Migration Invasion
DLC-1	STARD12 p122 RhoGAP	Cytoskeletal remodeling Rho GTPase activating protein	Motility Migration Invasion
E-Cadherin	CD324	Cell-cell and cell-matrix adhesion	EMT Invasion
FXR	NR1H4	Lipid and glucose metabolism Promote apoptosis	Invasion Colonization
GAS1		Cell cycle arrest Promote apoptosis	Colonization
GSN	ADF AGEL	Cytoskeletal remodeling Inhibit EMT	Migration
HUNK	MAK-V	Cytoskeletal remodeling	Motility Invasion
KAI1	CD82 SAR2 TSPAN27	Bind endothelial DARC (induce apoptosis) EGFR desensitization Up-regulate TIMPs Increase E-cadherin and β-catenin interaction	Intravasation \Transport
KISS1		Ligand for G-protein receptor (KISS1R) Angiogenesis Interact with PGC1α (cell metabolism)	Colonization Angiogenesis
LIFR	CD118 STWS	Activate hippo signaling	Migration Invasion Colonization
LSD1	KDM1	Chromatin remodeling	Invasion
MTBP	ACTFS HDMX HDM2	Cell cycle arrest Cytoskeletal remodeling Mitotic progression Chromosome segregation	Invasion
MetastamiR		Multiple functions (in general, reducing protein expression of metastasis promoters)	Migration Invasion EMT Colonization
MKK4 MKK6 MKK7 P38	MAPKK4 MAPKK6 MAPKK7 MAPKK14	Stress activated MAPK signaling	Migration Colonization
NDRG1	Drg1, Cap43	Promote cell differentiation Up-regulate E-cadherin	Angiogenesis Invasion

Table 1 (continued)

Metastasis Suppressor	Aliases	Proposed Function(s) [2]	Step(s) in metastasis inhibited [3]
	Rit42 RTP PROXY-1	Inhibit TGFβ mediated EMT	Colonization
Nm23	NDKB NME1	Inhibit activation of MAPK pathways Ras signaling Histidine kinase activity NDP kinase activity	Migration Colonization
OGR1	GPR68 GPR12A	G-protein coupled receptor signaling	Migration
RhoGDI2	ARHGDIB	Cytoskeletal remodeling Endothelin and Neuromedin U signaling Regulates Rho GTPases	Migration Colonization
RKIP	PFL0955C	Competitive inhibitor for RAF1-MEK interaction Cytoskeletal remodeling	Migration Invasion
RRM1	RIR1 RR1	Increases PTEN expression Decreases FAK phosphorylation Cytoskeletal remodeling	Motility Invasion
SSeCKs	AKAP12 GRAVIN	Scaffold for protein kinases Regulate Src,PKC and Rho signaling VEGF secretion	Angiogenesis Migration
Stefin A	CST6 EPM1 STFB	Cathepsin inhibitor	Angiogenesis Migration Invasion
TIMP	TIMP1 TIMP2 TIMP3 TIMP4	Inhibit MMP expression and signaling	Angiogenesis Migration Invasion Transport

Adapted from Bohl et al. [13]

MKK4, MKK7, and p38

Maintaining a delicate balance between ERK and p38 activation, the MAP Kinase Kinase proteins (MKKs) dictate a stress-activated protein (SAPK) signaling pathway that can lead to dormancy and proliferation. In response to stress signals from the environment, MAPKKK is activated, which can, in turn, activate MKK4, MKK7, or MKK3/MKK6 [159]. Activation of MKK4 leads to phosphorylation of p38, which can lead to arrest of cell cycle, apoptosis, and ultimately dormancy by suppression of cell growth [159, 160]. It is important to note that this effect is not seen in all cancer models, and the effect of p38 is likely resultant of microenvironment interactions with the disseminated cancer cells. Alternatively, MKK4 and MKK7 phosphorylate JNK1 (c-Jun N-terminal kinase) as another prong of the SAPK pathway. This pathway can lead to activation of SMADs, p21, p53, and numerous mitochondrial proteins involved in apoptosis and cell dormancy. Activation of p38 or JNK can both achieve cell dormancy as long as MKK4 or MKK7 kinase activity is preserved. When expression of MKK4 is lost, cells resume a metastatic phenotype and begin to proliferate in secondary tissue [161]. A loss of MKK4 expression in clinical settings tends to correlate with poor patient prognosis, while high amounts of phosphorylated (active) MKK4 tends to correlate with better patient outcome, suggesting an important role for MKK4 activation to maintain metastasis suppression [162].

Cell Adhesion Molecule/Tumor Suppressor Lung Cancer 1 (CADM1, TSLC1)

CADM1 TSLC1 is descriptively named for its role in cell-to-cell adhesion. An immunoglobulin superfamily member, CADM1 is responsible for eliciting adhesive properties in human epithelial cells, particularly mammary tissues [163]. Originally thought of as a tumor suppressor, CADM1 can attenuate primary tumor growth in some cancer lines, including non-small cell lung cancer and breast cancer [164].

True to its role as a metastasis suppressor, CADM1 expression does not alter tumor cell-autonomous properties involving proliferation or invasion [165]. Rather, CADM1 requires the host's adaptive immune system, primarily T-cells, to suppress metastasis [165]. CADM1 expression is regulated largely by promoter hypermethylation, leading to loss of CADM1 expression and a more motile, EMT phenotype in metastatic lesions [166–168].

Farnesoid X Receptor (FXR)

A nuclear hormone receptor, FXR, is a part of a receptor superfamily with four known isoforms which are found primarily in liver, intestine, kidney, and adrenal tissues. Under normal conditions, FXR is primarily a bile acid receptor which can bind sodium deoxycholate, responsible for bile acid and lipid homeostasis. Upon activation, FXR dimerizes with RXR to bind DNA and promote differential transcription. A main target of FXR is NDR2, which is up-regulated in response to FXR binding to suppress metastasis [169]. Recent reports have shown that FXR can bind sodium deoxycholate secreted from bone, which prevents the migration by inducing apoptosis, increasing uPA, and the formation of f-actin [170]. All of these results implicate a place for FXR as a metastasis suppressor, but the in vitro data is at odds with clinical data in some cancers. For example, FXR expression in pancreatic and esophageal cancers is correlated with poor patient prognosis [171–173]. As with other metastasis suppressors, designation in this category is cell type dependent.

Conclusions and Perspectives

Since discovery of the first metastasis suppressor in the mid-1980s, the family of molecules has steadily grown in size and insights into their respective mechanisms of action have increased concurrently. However, much remains to be learned. At an intuitive level, the conclusion that metastasis suppressors act at the interface between a tumor cell and each microenvironment is logical, since by definition, expression of metastasis suppressors allow growth in some tissues, but not others. At this boundary, metastasis suppressors receive and/or transmit signals to or from tumor cells. This regulatory circuitry is as complex as the process of metastasis itself. Multiple convergent and divergent pathways impinge upon myriad molecules, making it difficult to sort out primary from secondary from tertiary and higher order cellular changes.

Even this relatively superficial examination of metastasis suppressor function highlights several points. First, many, if not most, metastasis suppressor functions are cell-type dependent. While many metastasis suppressors can exert effects across multiple cell types, others do not. One must be careful not to extrapolate findings from one tumor type to another without support from experimental data.

Second, metastatic tumor cells are master manipulators of the microenvironment. Numerous examples where metastasis suppressors affect metabolism, stem cell mobilization, immune cell function and the extracellular matrix represent ways in which cancer cells customize tissues. In doing so, it is critical to distinguish tumor effects from host effects when studying cancers in situ.

Third, since metastasis suppressor expression affects how tumors respond to extrinsic signals, it can be posited that their expression and/or post-translational modification can vary, depending upon the microenvironment in which cancer cells find themselves.

So, while metastasis suppressor expression in circulating tumor cells appears to be offering some prognostic information, substantially more research will be required to make definitive conclusions. Likewise, recent data showing the transient nature of an epithelial-to-mesenchymal and mesenchymal-to-epithelial transition can occur in transit [174, 175], so too can one expect that some of the metastasis suppressor genes' expression may change in the transit compartments of the blood or lymphatic vasculature.

Finally, metastasis suppressors offer hope for improved prognostics as well as providing new targets for therapeutic intervention. The former are beginning to be seen in ever-growing clinical studies. Likewise, re-expression of metastasis suppressors may provide biomarkers from which therapeutic efficacy can be determined (Table 1).

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