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Metastasis Suppressors and Their Roles in Breast Carcinoma

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Abstract

Metastasis remains the most deadly aspect of cancer and still evades direct treatment. Clinically and experimentally, primary tumor development and metastasis are distinct processes—locally growing tumors can progress without the development of metastases. The discovery of endogenous molecules that exclusively inhibit metastasis suggests that metastasis is an amenable therapeutic target. By definition, metastasis suppressors inhibit metastasis without inhibiting tumorigenicity and are thus distinct from tumor suppressors. As the biology underlying functional mechanisms of metastasis suppressors becomes clearer, it is evident that metastasis suppressors could be harnessed as direct drug targets, prognostic markers, and to understand the fundamental biology of the metastatic process. Metastasis suppressors vary widely in their cellular localization: they are found in every cellular compartment and some are secreted. In general, metastasis suppressors appear to regulate selectively how cells respond to exogenous signals, by affecting signaling cascades which regulate downstream gene expression. This review briefly summarizes current functional and biochemical data on metastasis suppressors implicated in breast cancer. We also present a schematic integrating known mechanisms for these metastasis suppressors highlighting potential targets for therapeutic intervention.

Keywords

Breast cancer; Cancer; Metastasis; Metastasis suppressor; Signaling; Tumor; BRMS1; Cadherins; CD44; DLC1; Drg1; Gelsolin; KAI1; KISS1; MKK4; Nm23; RhoGDI2; RKIP; RRM1; SSeCKS

Cancer Metastasis

Metastasis continues to be the major cause of morbidity and mortality among cancer patients. Five-year survival rates for breast cancer drop from close to 100% when the cancer is localized to less than 25% when the cancer has colonized distant sites, underscoring the need to identify and target mediators in the metastatic process. Most therapeutic strategies include excision of the primary tumor with the anticipation that accompanying pharmaco-, radio-, and/or immunotherapy will eradicate any remaining tumor cells that have escaped surgical removal or that may have already disseminated. Although this approach is intuitive and has been effective in many cases, re-emergence of tumors and metastases is not uncommon. In fact, recurrent metastatic growth in breast cancer has been shown to occur years after the patient has been declared cancer free [1,2]. Approximately one-third of all breast cancer patients are positive for metastatic disease at the time of initial diagnosis [3]. This indicates that a sub-population

of tumor cells had disseminated, survived, resisted therapy, and had been dormant at secondary sites only to recommence growth. Transformation from a non-malignant to a metastatic phenotype is an evolutionary process whereby cancer cells progressively acquire characteristics that result from, or are a consequence of, selection of a sub-population of cells that are eventually able to complete all steps of the metastatic cascade [4]. Heterogeneity as a consequence of genomic instability assures that different populations within the tumor will have acquired differing characteristics during tumor progression. Whether the tumor cell is inherently “hardwired” to metastasize as a result of early acquired mutations or whether the metastatic phenotype is a result of natural selection during tumor progression is still open for debate [4-7].

Primary tumor formation is a necessary requirement for metastasis and it is estimated that approximately 1×10^6 cells escape into circulation per gram of primary tumor per day [8]. Only a fraction of cells leaving the primary tumor survive in circulation and even fewer cells colonize secondary sites [9]. The development of a metastasis, then, involves growth of such surviving cells at a secondary site. A unified definition of a “metastasis” is however still lacking and widely differs from a single disseminated cell (so-called micrometastasis) to a macroscopic mass that grows discontinuous from the primary tumor mass. We have advocated that disseminated cells can be called metastases only if they seed and proliferate at secondary sites [6]. The arrival of a tumor cell at the secondary site implies that the cell has completed all antecedent steps in the metastatic cascade (reviewed in [10]). However, seeding at ectopic sites is by no means an indicator or guarantor of growth. Once disseminated, tumor cells are believed to be regulated by their immediate microenvironment, although there is emerging evidence that preconditioning of seeding sites may occur before tumor cells reach the secondary site. That cancer cells prepare a “fertile ground” for their arrival was elegantly demonstrated by Kaplan et al. [11] who showed that melanoma cells released soluble factors that stimulated lung fibroblasts to secrete fibronectin creating an attachment site for the arrival of $\alpha 4\beta 1/\alpha 4\beta 7$ integrins, vascular endothelial growth factor receptor (VEGFR) positive progenitor hematopoietic progenitor cells. These progenitor cells then secreted metalloproteinases which released from the matrix a gradient of factors, including stromal derived factor-1 (SDF-1) that attracted chemokine (C-X-C motif) receptor 4 (CXCR4) positive tumor cells [11].

Formation of a tumor mass at the secondary site must follow some of the same steps implicit in primary tumor growth, including proliferation, evasion of apoptosis, and angiogenesis. However, the major distinction is that these disseminated cells grow in ectopic environments. Therefore, only those cancer cells [“seeds”] that are able to adapt to the new environment [“soil”]; those that already possess the ability to respond to microenvironmental signals at the “foreign” site; or those cells that can modify the new microenvironment can be expected to thrive. This “seed and soil” hypothesis was first presented by Sir Stephen Paget when he noted that breast cancer metastases followed a pattern of growth in specific organs, including bone and liver but not others, and reasoned that such patterning may have occurred due to inherent properties of the “soil” [12]. Indeed, the proclivity of cancer cells to grow at particular sites cannot be explained by the anatomy of the vasculature and associated hemodynamics alone. Micro-vasculature does play an important role in trapping disseminated cells within organs, however, this entrapment may not always translate to growth in situ [9,13]. Hart and Fidler confirmed Paget’s hypothesis of non-random tumor cell growth by implanting kidney, lung, and ovary tissue fragments in the thighs of mice and showed that B16 melanoma cells injected intravenously grew only in implanted lung or ovarian tissue [14]. These seminal experiments also demonstrated that disseminated cells arrested in the implants at essentially similar numbers but still showed organ selectivity for growth. Therefore, the role of vasculature in organ-selective tumor seeding and growth, although important, may be only secondary to the properties of the secondary site and the tumor cell itself.

Thus, while it can be reasoned from these data that the secondary microenvironment greatly influences eventual tumor cell growth, disseminated cancer cells must also be able to respond to the microenvironment in order to survive and grow. Hence, in theory, modulating the ability of cancer cells to respond to their microenvironment or modulating the secondary growth environment itself would be expected to alter metastasis. Collectively, the above data also reinforce the notion that metastasis is an interactive process—the tumor cell must be responsive to its particular microenvironment and the microenvironment must provide for the tumor cell to settle and eventually proliferate.

Modulation of Metastasis

The process of metastasis has theoretically been divided into a number of discrete steps including formation of the primary tumor, angiogenesis, invasion, intravasation, survival in the vasculature, extravasation, and colonization of the secondary site [10,15]. Every step in the metastatic cascade must be completed for successful manifestation of metastasis. Therefore, the expression or loss of any gene/protein that interferes with any of the step(s) in the metastatic cascade can, in theory, modulate metastasis. Progression towards a metastatic phenotype requires a concerted effort between a variety of molecules such as MMPs, oncogenes, cell adhesion molecules, etc. that have been implicated in advancing one or more steps of the metastatic cascade. In contrast, since metastasis is a linear process, blockade of any single step in the cascade would prevent metastasis [16]. Metastasis suppressors are a class of proteins that block metastasis without inhibiting primary tumor formation. Different metastasis suppressors disrupt one or more steps in the metastatic cascade. Thus, metastasis can potentially be blocked by restoring expression of a single molecule. For the most part, metastasis suppressors have been localized to chromosomal regions frequently lost in late-stage cancers and have traditionally been identified using differential expression analysis between metastatic and non-metastatic cells for a particular cancer type. As mechanisms underlying metastasis suppressor function become clearer, metastasis suppressors can be segregated into discrete functional groups (reviewed in [17]).

Metastasis suppressors exhibit diverse cellular localization complementary to their diverse cellular functions. For example, KAI1 is localized to the cell membrane and is believed to modulate tumor cell engagement with the vasculature; MKK4, Nm23, and RKIP localize to the cytoplasm and alter transmission of cellular signals; BRMS1 localizes mainly to the nucleus and is believed to alter gene expression as part of the mammalian Sin3: histone deacetylase complexes (mSin3:HDAC) altering levels and activity of signaling molecules including phosphoinositides and nuclear factor kappa B (NF- κ B). Within the cell, KISS1 co-localizes with the Golgi apparatus and is eventually secreted. Both these events are believed to be part of the metastasis suppressor action of KISS1. With restored expression of the metastasis suppressor KISS1, cells reach the secondary site without formation of overt metastases, suggesting that at least some metastasis suppressors may regulate growth at the secondary site. Thus, metastasis suppressors exhibit diversity of mechanisms of action and diversity in the steps of the metastatic cascade at which they act.

Necessary Qualifications for Being Classified as a “Metastasis Suppressor”

Metastasis suppressors suppress metastasis without inhibiting growth of the primary tumor. This criterion defines a unique class of metastasis modulators that are fundamentally different from tumor suppressors and which utilize distinct mechanisms for metastasis suppression. Thus, while tumor suppressors inhibit metastasis by virtue of inhibiting primary tumor growth, metastasis suppressors primarily affect downstream events other than primary tumor formation.

Literature exists in which molecules have been classified as metastasis suppressors based solely on in vitro data or even when primary tumor formation was suppressed [18-20]. It must be emphasized that while individual steps of the metastatic cascade may be modeled in vitro with a fair degree of physiological correlation, metastasis cannot be modeled in vitro due to the insufficient complexity of in vitro systems. Studies are also hampered by our incomplete understanding of mediators and mechanisms governing metastasis. Therefore, while demonstration of decreased invasive potential, anchorage independent growth, angiogenesis or proliferation can be used as preliminary screens for identifying candidate metastasis suppressors, these in vitro assays, either alone or in combination, are insufficient predictors of in vivo metastatic behavior.

Another question is, if the absence of a particular molecule increases metastasis, does that molecule qualify as a metastasis suppressor [21]? Although in theory, it is tempting to visualize such a molecule as a metastasis suppressor, unless experimentally proven according to the set criteria, such arbitrary classification will only add to confusion in literature and should be avoided.

Outlined below are metastasis suppressors that have been demonstrated to have an association with breast cancer metastasis. Although the precise mechanism of metastasis suppression are unknown for most, their currently known biochemical mechanisms relevant to metastasis are discussed (Tables 1, 2 and Fig. 1). Readers are encouraged to consult the primary literature for experimental details.

BRMS1

Metastasis suppression of MDA-MB-435 breast carcinoma cells following introduction of human chromosome 11 provided the first evidence for the presence of a metastasis suppressor on chromosome 11 [22]. BRMS1 (Breast Cancer Metastasis Suppressor 1) was subsequently identified by differential display in metastatic and metastasis suppressed MDA-MB-435 cells [23].

Experimentally, BRMS1 suppresses metastasis of breast, melanoma, bladder, and ovarian carcinoma cell lines [24-26]. However, apparently conflicting clinical data exist confounding the role of BRMS1 as a metastasis suppressor in breast cancer [27-30]. A limitation of the conflicting data appears to be the utilization of mRNA instead of protein as a measure of BRMS1 expression. The lack of antibodies against BRMS1 required the use of mRNA as a measure of BRMS1 expression in earlier studies. However, with the development of antibodies it appears mRNA and protein levels do not always correlate for BRMS1. In fact, our laboratory has observed increased BRMS1 mRNA without increased protein expression in the MCF10 breast cancer progression series (Hurst and Welch, unpublished data). Further, BRMS1 protein is stabilized by heat-shock proteins [31] and the eventual stability and localization of the BRMS1-containing complex is responsible for protein function and activity.

As with Nm23, early efforts to define BRMS1 function were to characterize its interacting proteins. Yeast two-hybrid studies revealed that BRMS1 interacted with retinoblastoma binding protein 1 (RBBP1; also known as ARID4A) and suppressor of defective silencing 3 (SUDS3; also known as mSDS3, SAP45) [31,32]. Both interactors are components of the core mSin3:HDAC co-repressor complexes. mSin3:HDAC complexes regulate gene transcription by recruiting specific HDACs to regulatory sequences and controlling accessibility of transcription elements by chromatin modification. Traditionally, the mSin3: HDAC complex is thought to regulate transcription through HDAC1 and 2. BRMS1 was also found to interact with cytoplasmic HDACs 4, 5, and 6, suggesting that BRMS1 may recruit other HDACs to the core mSin3:HDAC complex to execute transcriptional repression. Activity of BRMS1 is

dependent on its protein stability and the chaperone protein Hsp90 was found to stabilize BRMS1 protein [31].

Information obtained from interaction studies has greatly aided in the study of signaling mechanisms of BRMS1. The involvement of transcription factors as possible BRMS1 targets was deduced from its association with the mSin3: HDAC complex. The transcription factor NF- κ B is known to regulate transcription of multiple pro-tumorigenic and pro-metastatic genes in human cancer. Consistent with its function as a co-repressor, BRMS1 reduces transcription by promoting the association of HDAC1 with the p65 subunit of NF- κ B and decreases NF- κ B transcriptional activity by deacetylation of p65 [33,34]. Reduced NF- κ B activity causes decreased expression of osteopontin (OPN), a molecule implicated in breast cancer metastasis to bone [35]. BRMS1 also selectively modulates levels of phosphatidyl inositol (4,5) biphosphate (PtdIns(4,5)P₂), the primary substrate for phosphatidyl inositol 3-kinase (PI3K) in the PI3K-AKT pathway, which in turn, activates NF- κ B downstream of AKT [36]. Downregulation of two vital components of the same signaling cascade suggests an uncanny pathway-selective regulation by BRMS1 [33,37]. Such specific and redundant regulation by BRMS1 may be important in moderating responses from external growth factors and therefore growth at ectopic sites. Indeed, BRMS1 expression has been shown to be inversely correlated with human epidermal growth factor receptor 2 (HER2) expression in clinical samples [29, 30], strengthening the argument in favor of a growth-factor response regulatory role for BRMS1. Further, BRMS1 restores homotypic gap-junctional intercellular communication in metastatic cells and reduces colony formation in soft agar without alteration of either in vitro cell proliferation or invasion, suggesting a context-dependent regulation of tumor cell behavior [38].

As with other metastasis suppressors, the mechanism for differential regulation of tumor growth at orthotopic versus ectopic sites by BRMS1 remains unknown. Further, regulation of BRMS1 expression remains to be uncovered, and such knowledge may pave the path for the development of specific drugs that induce BRMS1 expression. That the BRMS1 gene is wild-type in all metastatic cells examined to date (Edmonds and Welch, unpublished data) and in limited clinical studies argues that treatment strategies employing restoration of endogenous expression may be a viable option.

KAI1

KAI1 (*kang-ai* 1; also known as CD82/C33/TIP30) was first identified following introduction of human chromosome 11 in rat prostate cancer cells by microcell-mediated chromosome transfer [39,40]. KAI1 is part of the tetraspanin superfamily which includes 32 known members in mammals alone [41]. Tetraspanins organize and stabilize large molecular networks that support a diversity of signaling and function (reviewed in [41]). Consequently, these proteins are implicated in various cellular processes, including motility, invasion, and cell signaling, among others [42-45].

KAI1 expression reduces metastasis in breast, melanoma, and prostate cancer models [40, 46-48]. Studies have also shown a loss of KAI1 in poorly differentiated and late-stage cancers, and inverse correlations have been demonstrated between the loss of KAI1 and prognosis in breast [49], colorectal [50], ovarian [51], cervical [52,53], oral [54], and non-small cell lung cancers [55]. This observed downregulation does not appear to involve KAI1 mutation or allelic loss and suggests transcriptional regulation [56].

Most studies with KAI1 have been performed with T-cells and, through these studies, a variety of mechanistic details have been uncovered. KAI1 interacts with a variety of cell surface molecules, including integrins [57-59], other tetraspans [60-62], major histocompatibility complex class 1 (MHC1) and human leucocyte antigen-DR (HLA-DR) [63,64], and

immunoglobulins. These interactions appear to modulate cellular adhesion, motility, and invasion [45,65] of tumor as well as immune cells. KAI1 and its interactions with the cytoskeleton through integrins are well characterized [62,66]. Palmitoylation of KAI1 appears to be essential not only for activation of p130CAS-CrkII signaling through the small GTPases (Rac, Rho, cdc42) and possibly their ability to effect cytoskeletal rearrangements, but also for the regulation of endocytosis of epidermal growth factor receptor (EGFR) [44,67,68]. Indeed disruption of palmitoylation disrupts motility and invasion—outcomes of downstream signaling through EGFR and the small GTPases, possibly suggesting a mechanism for KAI1-mediated metastasis suppression through the control of cellular response to external signals.

The most compelling mechanism demonstrated to date for KAI1 suggests an interaction between DARC (Duffy antigen receptor for chemokines)/gp-Fy on vascular endothelial cells and KAI1 on circulating tumor cells [69]. Although DARC is a seven-transmembrane spanning protein and binds multiple chemokines, downstream signaling does not appear to involve G-protein coupling [70]. DARC is expressed on the vascular endothelium of many organs, on erythrocytes, and epithelium [70,71]. DARC interacts directly with KAI1 on circulating tumor cells, rendering them senescent through the induction of p21 and downregulation of the senescence opposing gene *TBX2*. Further, KAI1 also interacts with integrins which may be used to tether tumor cells to the vasculature, thus possibly promoting an interaction between KAI1 and DARC [69]. Together, these results suggest a multi-modal inhibition of metastasis by KAI at different steps of the metastatic cascade.

Multiple mechanisms have been proposed to explain the regulation of KAI1 expression. Marreiros and colleagues demonstrated in vitro that the synergism between p53 and junB along with activating protein 2 (AP2) function was essential for KAI1 expression [72]. Kim and colleagues propose that KAI1 expression is dependent on a balance between the expression of Tip60 and β -catenin-reptin complexes [73]. Several lines of evidence argue for and against the proposed mechanisms, suggesting a more complex or possibly cell- and cancer-type specificity in regulation of KAI1 expression [57,74]. Phorbol myristate acetate (PMA) and etoposide have been shown to induce expression of KAI1 in vitro. Akita and colleagues showed that induction of KAI1 expression through PMA is mediated by protein kinase C [75]. On the other hand, induction of KAI1 expression through etoposide is mediated through p53 and c-Jun in concordance with previous reports suggesting a possible pathway for upregulation of KAI1 in vivo [57]. Treatment with etoposide also reduced invasion, however, etoposide is a topoisomerase II inhibitor and shown to induce cell cycle arrest and induce apoptosis. It is therefore difficult to dissect and separate mechanisms of decreased invasion and attribute the phenotype to KAI1 alone. Genes controlling metastasis suppressors have been found to be epigenetically regulated, and restoration of expression through reversal of promoter demethylation offers a possible pathway for inducing expression. Analysis of the KAI1 promoter revealed the presence of a non-hypermethylated CpG island. Predictably, treatment with 5-aza-2-deoxycytidine or trichostatin A did not upregulate KAI expression, suggesting other regulatory mechanisms [76].

KISS1

Introduction of chromosome 6 in metastatic melanoma cells suppressed metastasis to the lung and lymph nodes (>95%) without inhibiting tumor formation, suggesting the presence of a metastasis suppressor on chromosome 6 [77]. Interestingly, KISS1 was identified by subtractive hybridization and mapped to chromosome 1q32, suggesting regulation of KISS1 expression by gene(s) on chromosome 6 [78]. Further studies demonstrated that a co-factor required for Sp1 activity (CRSP3; mapped to chromosome 6) regulated thioredoxin interacting protein (TXNIP)/vitamin D upregulated protein 1 (VDUP1; mapped to chromosome 1), which, in turn regulated KISS1 expression [79]. Recent data indicate that a direct interaction between

the transcription factors AP2 α and Sp1 induces KISS1 expression [80,81]. These data provide a mechanism for the regulation of KISS1 and a potential explanation for its loss in breast cancer where AP2 α is frequently lost and in melanoma where CRSP3 is frequently deleted.

The metastasis suppressor function of KISS1 has been experimentally demonstrated in melanoma, ovarian, and breast cancer models [78,82,83] establishing KISS1 as a bona-fide metastasis suppressor in these models. Clinically, loss of KISS1 expression has been correlated with poor prognosis in thyroid, gastric, bladder, esophageal, and hepatocellular carcinomas along with melanoma and breast cancer (reviewed in [84]).

Independent laboratories have identified several cleavage sites in the amino acid sequence of KISS1 and subsequently the 54-amino acid processed form of KISS1 (KP54) was detected in human plasma samples, supporting the hypothesis that KP54 is likely to be secreted [85-87]. The fact that full length KISS1 could only be detected in cell lysates and that KP54 peptide fragments (kisspeptins) could only be detected in media from isolated trophoblasts strengthens the hypothesis that KISS1 is secreted and possibly processed at the cell surface [88]. The G-protein coupled receptor 54 (GPR54; also known as hOT7T175/AXOR12) has been identified as a cognate receptor for KP54. Activation of signaling through GPR54 abrogated SDF-1-induced AKT phosphorylation and chemotaxis, suggesting that the KISS1-GPR54 axis may be important in metastasis suppression [89].

Cells expressing KISS1 are metastasis suppressed even following direct intravascular injection, suggesting that the metastasis suppressor function of KISS1 lies at the secondary site [88,90]. Recent evidence indicates that secretion of KISS1 is required for metastasis suppression and tumor cell dormancy in the lung. Deletion of the secretion signal of KISS1 abolished its metastasis suppressor ability in C8161 melanoma cells. Further, re-expression of KISS1 in C8161 cells causes localization to the endoplasmic reticulum-Golgi; however, deletion of the secretion signal causes redistribution to the cytoplasm [88]. Surprisingly, cell lines suppressed for metastasis by over expression of KISS1 show little or no expression of GPR54, by real-time quantitative PCR, possibly suggesting a paracrine mechanism for KISS1 action or another, as yet, unidentified receptor. The mechanistic studies above were demonstrated in melanoma cells; however, key experimental results have been replicated in pilot studies using breast carcinoma cell lines as well.

A recent study showed that kisspeptins have potent vasoconstrictor abilities; this effect requires the presence of GPR54 [91]. While little difference in microvessel density was observed in primary tumors in KISS1-expressing versus non-KISS1-expressing melanoma and breast carcinoma cells, vessel *function* may have been reduced via a paracrine mechanism. How selective differences would occur with respect to growth at primary versus secondary sites is a question common to all metastasis suppressors. The answer may well ultimately reside in the analysis of various tumor microenvironments.

Activation of GPR54 by kisspeptins has been shown to induce activation of mitogen activated protein kinase (MAPK) signaling [89]. KISS1 has also been shown to decrease matrix metalloproteinase activity through the negative modulation of NF- κ B, suggesting activation of pathways independent of GPR54 involvement [92]. Overall, these data indicate that metastasis suppression requires secretion of KISS1 and possibly effects on vasculature at the secondary site, although the physiological relevance of myriad signaling pathways activated through the KISS1-GPR54 axis in metastasis suppression remains unclear.

As with other metastasis suppressors, the limited clinical studies to date show few mutations. That functional KISS1 was isolated from metastatic melanoma cells argues that the cellular defects of KISS1 in metastasis are largely due to defective regulation rather than mutation [93].

MKK4

MKK4 (Mitogen activated protein kinase kinase 4; also known as JNKK1/SEK1/MEK4/MAP2K4) was first identified as a metastasis suppressor in prostate cancer cells [94]. The MKK4 gene shows a low and consistent rate of mutation (5%) across tumor types, and loss of gene expression rather than mutation is believed to mediate metastasis [95]. Other than prostate cancer, MKK4 has been experimentally demonstrated to be a metastasis suppressor in ovarian carcinoma [96,97]. An inverse correlation has been demonstrated in a subset of brain metastases from breast cancer where MKK4 mRNA as well as protein levels were reduced along with other metastasis suppressors and also in pancreatic cancer where reduced MKK4 levels were seen in primary tumors as well as in lymph node metastases [98,99]. In contrast, Wang and colleagues showed that ectopic expression of MKK4 in a panel of breast and pancreatic cancer cell lines induced a “pro-oncogenic” phenotype in vitro opposite to that seen with clinical correlations and in prostate and ovarian carcinoma [100]. The role of MKK4 in breast and pancreatic cancer thus remains controversial and warrants further investigation.

MKK4 is a dual specificity kinase and is an intermediate of the stress-activated MAPK pathways. Three MAPK pathways are currently well-studied: (1) extracellular-signal regulated kinase (ERK), (2) p38, and (3) c-Jun NH2 terminal protein kinase (JNK; also known as SAPK). MKK4 is unique in serving as a common link between and as a dual activator of both p38 and JNK pathways in response to stress, growth factors, and cytokines. Once phosphorylated, both p38 and JNK phosphorylate downstream transcription factors, including c-jun, ATF2, and elk-1 (reviewed in [101]) resulting in an altered pattern of gene expression. These events are implicated in such cellular processes as apoptosis, differentiation, and proliferation [102]. Sustained activation of the upstream mixed lineage kinase 3 (MLK3) also activates MKK4 and downstream c-jun. Activation of c-jun acts as a negative feedback, preventing downstream activation of ERK through MEK [103]. Thus, activation of the JNK pathway downstream of MKK4 may prevent MAPK activation through various mitogenic stimuli and may contribute to cancer cell unresponsiveness towards growth factors.

The exact mechanism of MKK4 mediated metastasis suppression remains unclear. However, similar to the metastasis suppressor KISS1, cells with ectopic MKK4 expression complete all steps of the metastatic cascade and reach the secondary site but fail to form overt metastases [94]. MKK4 kinase activity is essential for metastasis suppression and is probably required to activate a particular transcription profile that mediates growth arrest or cell cycle alterations [104]. MKK6 can also activate p38, a downstream mediator of MKK4. Ectopic expression of MKK6 but not MKK7 (MKK7 activates JNK and not p38) in ovarian cancer cells also suppressed metastasis, indicating that stress-pathways selectively activating p38 may mediate metastasis suppression [97].

Nm23

The first metastasis suppressor discovered and the most widely studied, Nm23 (Non-metastatic clone # 23) was originally identified in murine melanoma cell lines of varying metastatic potential by differential colony hybridization [105]. The human *nm23* family now comprises eight members; however, to date only Nm23-H1 and Nm23-H2 have been experimentally demonstrated to suppress metastasis [106].

Nm23 expression has been studied in a variety of tumor types to determine clinical utility as a prognostic marker. Decreased expression of Nm23 was correlated with poor patient survival and tumor grade in subsets of breast, ovarian, hepatocellular carcinomas and melanoma, among other cancer types (reviewed in [107]). In contrast, increased Nm23 expression in neuroblastoma was correlated with poor patient prognosis and survival and was attributed to gene amplification. In addition to gene amplification, mutations in *nm23* have been noted in

neuroblastoma; however, the incidence is rare and has been observed only in late stage tumors [108]. Such disparity underscores the fundamental differences in biology of histologically distinct cancer types and argues caution when extrapolating findings from one type to another.

Establishment of metastasis involves cross-talk between the tumor cell and its microenvironment, and disruption of such communication would be expected to reduce metastatic potential. Consistent with this idea, transfection of Nm23 reduces in vitro motility and/or colony formation in response to a variety of growth factors, including transforming growth factor- β (TGF- β) [109,110], IGF-1, and PDGF [111]. Several studies in clinical specimens and in experimental systems have demonstrated an inverse correlation between Nm23 and cell surface receptors, such as EGFR [112] and HER2 [113-116] in multiple tumor types [110]. Although these studies point towards a potential mechanism of altered responsiveness towards exogenous growth signals in Nm23 mediated metastasis suppression, these data are only correlative and over interpretation should be avoided.

Another approach utilized to characterize the mechanism of metastasis suppression involved identification of Nm23-interacting proteins. Using co-immunoprecipitation and yeast-two hybrid analyses, Nm23 was associated with a large cohort of proteins with distinct functions including Tiam1 (nucleotide exchange factor for Rac1) [117], Rad (GTPase) [118,119], glyceraldehyde 3-phosphate dehydrogenase [120], vimentin [121], G-proteins [122], and casein kinase 2 (CK2) [123] among others (reviewed in [124]). Interaction with other proteins was presumed to either alter Nm23 function (e.g. CK2, GAPDH) or vice versa (e.g. Tiam1, Rad). However, the physiological relevance of many interactors remains speculative in most cases.

Nm23 has three reported activities: NDP kinase [125], histidine kinase [126] and exonuclease [127]. Mutants that disrupt all of the activities are unable to suppress metastasis. CK2 was reported to phosphorylate Nm23 and reduce its NDPK activity; however, metastasis suppression by Nm23 has been shown to be independent of its NDPK activity [128,129]. Further, Kaetzel and colleagues have also shown that point mutants that disrupt either the exonuclease activity alone or the NDPK activity alone fail to suppress metastasis in 1205Lu melanoma cells (Kaetzel, personal communication). Thus, the definitive mechanism of action for Nm23 remains uncertain.

Mechanistically, the most convincing lead appears to be the interaction of Nm23-H1 with the kinase suppressor of Ras (KSR). KSR is a scaffold protein that binds members of the MAPK pathway (Raf-1, MEK1/2, and ERK1/2) [130]. Under resting conditions, KSR is retained in the cytoplasm by binding to 14-3-3 proteins; this binding is mediated by Ser³⁹² phosphorylation of KSR. Mitogenic stimuli induce dephosphorylation of KSR, resulting in translocation to the plasma membrane and facilitation of signaling between Raf, MEK and ERK [131]. Nm23-H1 phosphorylates KSR at Ser³⁹², possibly sequestering KSR and preventing further downstream activation of the MAPK pathway. This hypothesis is strengthened by the observation that Nm23-H1 transfectants show reduced basal and stimulated MAPK phosphorylation [132].

The eventual aim is to utilize Nm23 and other metastasis suppressors in the clinical setting either as prognostic markers or as targets for drug development. To this end, devising strategies to restore expression and determining whether restored expression is sufficient to suppress metastasis in vivo remains a continual challenge. Some headway has been made with Nm23 with the identification of transcription factor binding sites (MAF/Ets, a CTF/NF1 half site and an ACAAAG enhancer) in the Nm23 promoter contributing to differential expression in breast carcinoma cells [133-136]. These promoter elements were determined to be identical to those in the MMTV-LTR and implicated in the regulation of MMTV through glucocorticoids. Both dexamethasone and medroxyprogesterone acetate (MPA) induced Nm23 expression in vitro,

and MPA treated mice showed a significant reduction in frequency and incidence of metastases, indicating possible manipulation of metastasis suppressor expression through external drug administration [136,137].

RKIP

Raf kinase inhibitor protein (RKIP) was first identified as a metastasis suppressor in prostate cancer [138]. Only correlative evidence of RKIP as a metastasis suppressor in breast cancer exists. RKIP was shown to be expressed in human breast tumors but largely absent in matched lymph node metastases [139].

Biochemically, RKIP functions as a negative modulator of MAPK signaling by preventing activation of MEK by Raf-1. Although Raf-1, MEK, and ERK associate with RKIP, neither are substrates of RKIP. RKIP primarily competitively inhibits Raf-1 interaction with MEK, indicating that its major function may be to sequester MEK and control downstream signal transmission by serving as a checkpoint [140]. Both Raf-1 and B-Raf are required for maximal activation of the MAPK pathway; however, apparently conflicting data exists regarding the ability of RKIP to inhibit B-Raf [141,142]. Such apparent contradiction may be a result of different cell line models used in these studies. Activation of the MAPK pathway prevents induction of apoptosis. In line with this paradigm, reduction of RKIP expression increases basal MAPK activation and confers resistance to apoptosis. Conversely, enforced RKIP expression sensitizes breast cancer cells to apoptosis [143].

A recent study indicates that RKIP mRNA binds and potentially activates 5'-phosphorylated, 2, 5'-linked oligoadenylates [144]. RNase L binds to these activated oligoadenylates and induces apoptosis through caspases [145]. These results provide a novel function for RKIP and possibly may be important in its metastasis suppressor function.

Tumor or Metastasis Suppressor, Depending upon Cellular Context

A number of candidate molecules suppress metastasis *in vivo*, however, they do not adhere to the strict definition of a metastasis suppressor, i.e., they suppress tumor formation in some models. Molecules that display such dual functionality (DLC1, DRG1) are outlined in Tables 1, 2 and in Fig. 1, but are not discussed further here because of space limitations.

There are a growing number of metastasis suppressors identified [146]. Since most have only recently been discovered, they have not been tested yet in breast cancer. Multiple cadherins are implicated in cell-cell adhesion and are thought to prevent the initial detachment of tumor cells from the primary site. Recently, Stupack and colleagues demonstrated that cellular adhesion to non-preferred substrates could induce caspase-8-dependent apoptosis [147]. In their neuroblastoma model, caspase-8 selectively inhibited metastasis without blocking tumor formation. CD44, the receptor for hyaluronic acid and osteopontin, has complex roles in cellular adhesion. Depending upon which of the many splice variants is studied, expression can modulate adhesion, cellular homing, metastasis and cellular growth. Gelsolin, which belongs to a superfamily of calcium-dependent actin-interacting proteins regulates cellular motility, but also contributes to regulation of cell division, invasion and adhesion.

Rho-guanine nucleotide dissociation inhibitor (RhoGDI2, also known as RhoGDI β , LyGDI, GDID4) was first identified as a metastasis suppressor in bladder cancer [148,149]. RhoGDIs sequester Rho proteins in an inactive state and transport them to effector sites. Two targets of RhoGDI2 are currently known, endothelin1 (ET1) and neuromedin U (NMU). Both ET1 and NMU promote metastasis; so, RhoGDI2 is suspected to regulate metastasis promoters [150, 151]. Interestingly, ET1 has been shown by Guise and colleagues to regulate metastasis in bone [152,153], the most frequent site of breast cancer metastasis.

Ribonucleotide reductase M1 (RRM1) converts ribonucleotides to deoxyribonucleotides required for DNA synthesis, but its mechanism of action in controlling metastasis is still unknown. Src-suppressed C Kinase substrate (SSECKS, pronounced essex) is the rodent ortholog of human A-kinase anchoring protein 12 (AKAP12)/Gravin. SSECKS is a major substrate for protein kinase C and also functions as a scaffolding protein for protein kinases A and C. Recent evidence indicates that re-expression of SSECKS inhibits expression of VEGF, suggesting a key role in regulating angiogenesis [154].

Perspective

Currently, the greatest challenges facing clinical cancer medicine are (1) to be able to detect cancer at the earliest possible stage and, (2) managing cancer once it has spread beyond the primary tumor. Early detection of tumors is extremely desirable since the tumor burden is relatively small, which not only decreases the statistical likelihood of metastatic cells developing within the primary tumor mass, but also means that fewer cells are likely to have escaped from the primary tumor. Both parameters correspond to a decreased likelihood of metastasis, in addition to a decreased likelihood of metastatic variants within a heterogeneous tumor. Early detection also decreases the chances of outgrowth of drug-resistant populations. The diagnostic challenge is largely a matter of limit of detection. Currently, even with the most advanced imaging techniques, small tumors escape identification in random screens. Although still in the developmental stage and as a possible future complement to imaging, it is now possible to detect minimal disease early through analysis of bone marrow aspirates and serum samples for genetic markers of tumor progression [155,156]. Early detection is desirable for all patients, but particularly those considered to be at “increased risk.” Also, early detection of disseminated cells before they escape dormancy should reduce the unnecessary adjuvant therapy for patients with exclusively localized tumors. For patients with micro-metastases, the considerations are more complex. The most convincing evidence that maintenance of dormancy may be clinically achievable was demonstrated in mice treated with medroxyprogesterone; these mice showed elevated Nm23 expression in metastases and a significant decrease in metastatic burden compared to controls [137]. Although these results are too preliminary to warrant clinical implementation, they nonetheless highlight potential clinical utility.

Adjuvant and neo-adjuvant chemotherapy are mainly aimed at metastases, if any. Cells that escape the initial adjuvant treatment can recur as metastases. Too often, such cells have further acquired a resistant phenotype or characteristics of cancer stem cells are manifest. Importantly, a recent study demonstrated that administration of the plant-derived sesquiterpene lactone—parthenolide to mice (using a murine osteosarcoma cell line LM8) led to lung metastasis suppression. However, in this model, subcutaneous injection of LM8 cells followed by treatment with parthenolide did not suppress tumorigenicity [157]. Conceptually, the drug mimics the activities of metastasis suppressors. While gene replacement approaches may ultimately be possible, the plausibility of using targeted molecules to achieve the same end point is more immediately feasible. In addition, granulocyte macrophage-colony stimulating factor (GM-CSF) is important for osteolytic breast cancer metastasis. Inhibition of GM-CSF suppresses bone metastasis without affecting primary tumor growth [158]. Although results with parthenolide and GM-CSF are preliminary, could these results point to potentially identifying “metastasis suppressor drugs?” Mechanistic studies with another metastasis suppressor RhoGDI2 uncovered NMU as a target and a novel metastasis promoter [151]. Studies of mechanisms underlying metastasis suppression therefore not only show the potential to identify novel “druggable targets” but also novel mediators in the metastatic process.

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Abbreviations

AKT	v-akt murine thymoma viral oncogene homolog 1
AP2	Activating protein 2
ARID4A	AT rich interactive domain 4A
CK2	Casein kinase 2
CRSP3	Co-factor required for Sp1 activity
CXCR4	Chemokine (C-X-C motif) receptor 4
EGFR	Epidermal growth factor receptor
ET1	Endothelin 1
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPR54	G-protein coupled receptor 54
GSK1	Glycogen synthase kinase 1

GTPase	Guanine tri-phosphatase
HER2	Human epidermal growth factor receptor 2
HLA-DR	Human leucocyte antigen-DR
IL8	Interleukin 8
JNK	c-Jun NH2 terminal protein kinase
KSR	Kinase suppressor of Ras
MAPK	Mitogen activated protein kinase
MHC1	Major histocompatibility complex class 1
MLK3	Mixed lineage kinase 3
MPA	Medroxyprogesterone acetate
HDAC	Histone deacetylase
NF-κB	Nuclear factor-kappa B
NMU	Neuromedin U
PI3K	Phosphatidyl inositol 3-kinase
PtdIns(4,5)P₂	Phosphatidyl inositol (4,5) bisphosphate
PMA	Phorbol myristate acetate
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RBBP1	Retinoblastoma binding protein 1
RhoGAP	Rho GTPase-activating protein
SDF-1	Stromal derived factor-1

SUDS3	Suppressor of defective silencing 3
TGF-β	Transforming growth factor- β
TXNIP	Thioredoxin interacting protein
VDUP1	Vitamin D upregulated protein 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor 1

Figure 1.

Schematic of proposed signaling by metastasis suppressors. *Blue (dark) shapes* indicate metastasis suppressors and *red (light) shapes* indicate signaling molecules. *Black (dark) arrows* indicate positive regulation and *red (light and stunted) connections* represent negative regulation. Cellular location of metastasis suppressors is according to currently known data. Only those biochemical pathways and interactions that have been experimentally confirmed are depicted, however, not all signaling pathways are shown (see text for details).

Table 1**Metastasis suppressors and proposed mechanisms**

Metastasis suppressor	Chromosomal location	Proposed mechanism(s) of metastasis suppression	Selected references
BRMS1	11q13.1–q13.2	Part of mSin3:HDAC complexes that regulate transcription, downregulates PtdIns (4,5)P ₂ and inhibits NF-κB activity and decreased osteopontin expression	[33,37]
Caspase8	2q33–q34	Induction of apoptosis through unligated integrins	[147]
E-cadherin, N-cadherin, cadherin -11	16q22.1, 8q11.2, 16q22.1	Cell–cell/cell–matrix adhesion	[159,160]
CD44	11p13	Binds hyaluronan and osteopontin	[161]
DLC1	8p22–p21.3	Rho-GTPase activating protein controlling cytoskeletal architecture	[162]
Drg1	8q24.3	Unknown	[163]
Gelsolin	9q33	Actin filament dissolution; reduced motility	[164]
KAI1	11p11.2	KAI1 on tumor cells interacts with DARC on vascular endothelium leading to tumor cell senescence	[69]
KISS1	1q32	Maintains tumor cell dormancy at secondary site	[88]
MKK4	17p11.2	Maintains dormancy at secondary site. May affect cell cycle by induction of p21Cip1/Waf1 and p53, activates p38 and/or JNK	[17,165]
Nm23	17q22	Phosphorylates KSR and prevents downstream activation of the MAPK pathway	[132]
RhoGDI2	12p12.3	Regulates Rho; negatively modulates endothelin 1 and neuromedin U expression	[150,151]
RKIP	12q24.23	Inhibits MAPK signaling by competitive inhibition of Raf-1:MEK interaction	[140]
RRM1	11p15.5	Upregulates PTEN; decreases FAK phosphorylation	[166]
SSeCKS	6q24–q25.2	Scaffold protein for protein kinases A and C, inhibits osteopontin, VEGF expression; upregulates vasostatin	[154,167]

Chromosomal locations have been sourced from the National Center for Biotechnology Information (NCBI) Entrez Gene database updated 01/08/2007.

Table 2

Metastasis suppressors and clinical correlations

Metastasis suppressor	Cellular localization	Cancer type	Expression	mRNA				Prognosis	Selected references
				Protein		Metastasis			
				Primary	Metastasis	Primary	Metastasis		
BRMS1	Nuclear	Breast	NT	NT	NC	NC	NT	[27,29,30]	
		Breast	NT	NT	Increased	NT	Favorable		
Caspase-8 E-cadherin, N-cadherin, cadherin-11	Cytosol	Breast	↓	NT	NT	NT	NT	Poor	
		Breast	Expressed	↓	Expressed	↓	NT	NT	[168]
	Cell membrane	Neuroblastoma	↓	NT	NT	NT	NT	NT	[169]
		Breast	↓	NT	NT	NT	NT	NT	NT
	Cell membrane	Prostate	↓	NT	↓	NT	NT	NT	[170]
		NT	NT	NT	NT	NT	NT	NT	NT
	Cytosol	Breast	↓	↓	NT	NT	NT	Poor	[171]
		Prostate	↓	↓	NT	NT	NT	Poor	
	Cytosol	NT	NT	NT	NT	NT	NT	NT	
		Breast	↓	NT	NT	NT	NT	NT	[49-51,55]
Gelsolin KAI1	Cell membrane	Colorectal	↓	↓	NT	NT	NT	NT	
		Cervical	↓	↓	↓	↓	NT	NT	
	Ovarian	NSCLC	↓	↓	NT	NT	NT	NT	
		Bladder	↓	↓	NT	NT	NT	NT	
	Golgi/secreted	Esophageal	NT	↓	NT	NT	NT	NT	
		Gastric	NT	NT	↓	NT	NT	Poor	[172-174]
	Cytosol	Hepatocellular	NT	NT	NC	NT	NT	NT	
		Thyroid	NT	NT	Expressed	Expressed	NT	NT	
	MKK4	Breast	Expressed	↓	Expressed	Expressed	NT	NT	[98,99]
		Pancreatic	↓	NT	↑	↓	NT	Inconclusive	
Nm23	Cytosol/nucleus	Breast	↓/NC	↓/NC	↓/NC	↓/NC	Poor	Reviewed in [107]	
		Hepatocellular	Expressed	↓/NC	NT	NT	Poor		
		Melanoma	Expressed	↓	NT	NT	Poor		
		Ovarian	Expressed	↓	Expressed	↓	Poor	[175]	
RhoGDI2 RKIP RRM1 SSeCKS	Cytosol Cytosol Cytosol/nucleus Cytosol	Bladder	↓	NT	NT	NT	Poor	[139]	
		Breast	Expressed	↓	NT	NT	Poor		
		NT	NT	NT	NT	NT	NT	NT	
		Expressed	NT	NT	NT	NT	NT	[176]	

NC No change, NT Not tested