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Metastasis Suppressors and the Tumor Microenvironment

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Abstract

The most lethal and debilitating attribute of cancer cells is their ability to metastasize. Throughout the process of metastasis, tumor cells interact with other tumor cells, host cells and a variety of molecules. Tumor cells are also faced with a number of insults, such as hemodynamic sheer pressure and immune selection. This brief review explores how metastasis suppressor proteins regulate interactions between tumor cells and the microenvironments in which tumor cells find themselves.

Keywords

Nm23; RECK; BRMS1; KISS1; Kisspeptin; TIMP; LSD1; CD44; OGR1; E-cadherin; JNKK1/MKK7/p38; RhoGDI2; DLC1; DRG1; gelsolin; KAI1; SseCKS/Gravin/AKAP12; HUNK; caspase 8; ribonucleotide reductase M1; angiogenesis; review

1. Introduction

Metastasis is the process in which neoplastic cells leave the site where a tumor formed, travel to nearby or distant discontinuous sites and proliferate into a macroscopic, clinically relevant mass(es) [1,2]. It has long been recognized that metastasis involves intrinsic (i.e., genetic) as well as extrinsic (i.e., tumor cell-microenvironmental signals) factors. Our objective is to explore the interface between specific genetic changes, specifically of metastasis suppressors, and the regulation by (or response to) the microenvironments with which tumor cells come into contact. Ultimately, the data presented here support the concept that several metastasis suppressors are key intermediaries between environmental signals and tumor cell response to environmental signals.

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Over a century ago, Stephen Paget articulated the interactions between tumor cells and as-yet-undefined factors in bodily tissues that determine whether disseminated cells successfully colonize those organs [3]. Abundant data support the so-called Seed-and-Soil hypothesis; however, even in 2011, the molecular underpinnings are still not well characterized [4]. Disseminated tumor cells «seeds» recognize surface molecules in various soils to preferentially adhere there. The ability of cells to successfully grow following dissemination is determined by their ability to respond to growth promoting factors and/or resist growth inhibitors secreted by various tissues or matrix-associated factors [5-8]. Despite not yet knowing all of the detailed molecular interactions, Stephen Paget presented an important insight regarding tumor-host interactions.

It is clear that tumor cell interactions are crucial at every step of the metastatic cascade. Every communication – even between two cells – contributes enormous complexity. Neoplastic cells are dependent upon genetic alterations that distinguish them from their normal counterparts, but are also exquisitely sensitive to extracellular factors that may, or may not, allow them to metastasize successfully. Tumor cells alter the microenvironment directly (e.g., proteolytic cleavage of the ECM) or indirectly (e.g., induction of nearby cells to secrete proteases). Reciprocally, tumor cells can be impacted by stromal cells (e.g., lymphatic or vascular) at each step in the cascade. Thus, it is important to focus beyond the genetic mutations, deletions or chromosomal translocations found within a tumor cell by recognizing that stromal factors are present and intimately involved in the process. What we learn from these interactions will help to provide insights that may allow development of new therapies targeting metastasis.

This review is based upon a simple tenet – some, if not most, of the genetic changes occurring within cancer cells either manipulate, or are manipulated by, the stimuli from the cells or matrices with which the tumor cells come into contact or the factors secreted by cells throughout a tumor cell's journey. This review is also submitted at a time when the field of metastasis research is evolving from a tumor cell-centric to a more holistic perspective that integrates both the neoplastic cell and the microenvironment. The breadth of the concepts cannot be covered in the space allowed. So, we will focus this review exclusively upon how changes in the expression of a new class of genes, metastasis suppressors, alter tumor microenvironmental changes.

2. Metastasis Suppressors

Metastasis suppressors are defined as molecules whose expression results in the inhibition of a neoplastic cell's ability to metastasize while having little effect on primary tumor growth (reviewed in [1]). This family of molecules was first described in 1986, with the discovery of *Nm23*. Since then, more than thirty metastasis suppressors have been identified based upon this functional definition [1]. Still, the mechanisms of action for most are not yet known despite concerted efforts by many laboratories. It is clear, however, that metastasis suppressors are found both within cells and in the extracellular milieu, that their mechanisms of action are diverse, and that each regulates different steps of the metastatic cascade (reviewed in [1]). The metastasis suppressors discussed below are organized by cellular localization and functional class.

2.1 Cell Surface Adhesion Molecules and Receptors

2.1.1 E- and N-Cadherins—Epithelial cell-cell interactions are mediated by cadherins, transmembrane glycoproteins that form Ca^{+2} -dependent homotypic complexes [9]. In many tumor types, E-cadherin loss occurs during epithelial-to-mesenchymal transition (EMT, reviewed in [10-12]), which correlates with invasion and metastasis. Collectively, loss of E-cadherin is thought to be causative for invasion. E-cadherin expression can be regulated at

transcriptional and post-transcriptional levels. In particular, the zinc finger transcriptional repressors Snail and Slug have been implicated in repressing E-cadherin transcription. Cadherins are regulated by catenins (α , β , γ , and p120 catenins), cytoplasmic proteins that functionally link cadherin complexes to cytoskeleton. β -catenin is both a cell adhesion protein and a transcription factor.

Another putative metastasis suppressor is N-cadherin which, when ectopically expressed in an osteosarcoma line, inhibits pulmonary metastasis [13]. However, contradictory data showing that N-cadherin increases aggressiveness and metastasis in breast and melanoma cell lines raise questions regarding calling it a metastasis suppressor [14,15].

As cells growing in an epithelial layer decrease cohesion, they often produce cellular protrusions through reorganization of the cytoskeleton and plasma membrane that form new contacts with the surrounding microenvironment [1,2,34]. These new adhesive contacts promote migration through ECM. These observations also contribute to the conclusion that cancer cells, as they progress, become more autonomous and less reliant upon other cells and specific matrix interactions for survival signals.

It is important to emphasize, however, that EMT is not required for invasion or metastasis. Many carcinomas migrate as clusters of cells, suggesting that they have maintained vestigial epithelial-epithelial interactions (perhaps, though not directly tested to our knowledge, maintaining N- or E-cadherin expression). The EMT-based motility emphasizes migration of individual cells in a manner dependent upon proteolysis; whereas, individual cell movement independent upon proteases – the so-called epithelial-amoeboid transition – is becoming increasingly recognized [16].

Clearly, more work is required to define how cadherins play a role in metastasis suppression. Nonetheless, it is clear that different cadherins will play distinct roles in different tissues. This highlights an emerging theme in the metastasis suppressor field – context is critical.

2.1.2 KAI1—Kang-Ai1 (Chinese for anti-cancer; also known as CD82/C33/TIP30) was first identified in rat Dunning prostate cancer cells [17]. As for many of the metastasis suppressors, evidence for their existence was suggested by non-random losses of chromosomal material in late-stage cancers. For KAI1, human chromosome 11 was introduced into the metastatic prostate variants by microcell-mediated chromosomal transfer (MMCT). Chromosome 11-containing hybrids were significantly suppressed for metastasis without preventing primary tumor formation. Positional cloning identified KAI1 at band 11p11.2 [17-19]. Following demonstration that KAI1 inhibited metastasis of prostate cancer cell lines, it was also shown to suppress metastasis in breast and melanoma cells [20,21]. Consistent with its role as a metastasis suppressor, KAI1 expression is frequently down-regulated during progression of multiple tumor types, including prostate [17], breast [22,23], colorectal [24], ovarian [25], cervical/endometrial [26,27], oral [28] and non-small cell lung [29].

Tetraspanins, including KAI1, interact with other signaling molecules. KAI1 interacts with other tetraspanins, immunoglobulins, integrins and histocompatibility molecules, including the epidermal growth factor receptor which instigates EGFR endocytosis and migration signals. However, the most convincing studies regarding a mechanism of action for KAI1 came from the laboratory of Watabe and colleagues.

KAI1 directly interacts with DARC (Duffy antigen receptor for chemokines)/gp-FY) on vascular endothelial cell surfaces to induce tumor cell senescence [30] when cells have

intravasated. The story remains incomplete because senescence has been shown to occur even in the absence of KAI1-dependent activation of DARC signaling [31]. Collectively, their model proposes that KAI1-expressing cancer cells grow and invade locally, but upon intravasation and interaction with DARC-expressing endothelial cells, the tumor cells cannot complete subsequent steps in the metastatic cascade. Interestingly, KAI1-expressing melanoma cells injected into DARC knockout mice developed significantly more lung metastasis than in wildtype mice [30]. Thus, KAI1::DARC interaction-dependent metastasis regulation is controlled by the physiological balance of communication between the two molecules.

2.1.3 KISS1R—Although discussed in more detail below in the context of secreted metastasis suppressors, the KISS1 receptor (KISS1R, also known in the literature as GPR54, AXOR12, hOT7T175) appears to be involved in metastasis suppression in some tumor cells. KISS1R is a G-protein-coupled receptor (GPCR) that is expressed almost ubiquitously at low levels, but is abundantly present in specialized neurons within the hypothalamus, pituitary and arcuate nucleus, where it is responsible for regulating pubertal development in the hypothalamic-pituitary-gonadal axis [32]. KISS1R bind internal fragments derived from KISS1, one of the first described metastasis suppressors. Initially, this interaction is thought to form an autocrine loop by which the tumor cells suppressed metastasis. However, an autocrine loop has not been formally established in any cell line that has not been transfected with the receptor (Beck and Welch, 2010). Recent as-yet unpublished data from our laboratory establish a paracrine feedback mechanism in which KISS1 secreted by tumor cells initiates secretion of growth inhibitory signals, depending upon the origin of the stromal cells (Beck, B.H. and Welch, D.R., unpublished observations). These findings illustrate how melanoma cells expressing KISS1 might be able to grow in the skin, but fail to grow after they have already disseminated [33].

2.1.4 CD44—CD44 is a trans-membrane glycoprotein that binds extracellular matrix components such as hyaluronic acid and the pro-metastatic factor, osteopontin. CD44 expression modulates adhesion, lymphocyte homing and activation. Loss of CD44 expression tends to occur in high grade tumors and metastases. Depending upon tumor type, the cell line used, and the model being evaluated, CD44 expression can increase tumorigenicity and/or metastasis as well as functioning as a metastasis suppressor [34,35].

In recent years, the notion of so-called cancer stem cells has been popularized and many have postulated that the CSC may be responsible for metastasis (reviewed in [36]). CD44 surface expression is a marker for cancer stem cells [37-39], further suggesting a potential role(s) of CD44 in metastatic behavior. However there remains a significant amount of ambiguity regarding the metastasis-promoting or -suppressing effects of CD44 because the molecule is highly post-transcriptionally regulated in addition to numerous splice variants that are expressed in a cell-dependent manner. Thus, some of the apparently contradictory data may be resolved when it is possible to unambiguously identify functions for each of the splice variants.

2.1.5 OGR1—Ovarian cancer G-protein coupled receptor (OGR1, also known as GPR58) is another GPCR metastasis suppressor in prostate carcinoma cells [40]. Relatively little is known about this molecule and its mechanism of action has been speculated based upon the roles of related family members in mediating the functions of lysophospholipids [41]. OGR1 regulates endothelial barrier integrity, proliferation and tube formation and T-cell migration. There is also data suggesting that OGR1 and related family members have proton sensing properties. Over-expression of OGR1 appears to induce secretion of a hydrophobic factor that appears to be important for OGR1 anti-metastatic actions [41]. Since it is expressed on

the cell surface, it is presumed that OGR1 is involved in receiving or transmitting signals from the tumor cell; however, the nature of the signal(s) is not definitively known.

2.1.6 DCC1—Deleted in colon cancer (also known as UNC-40 or Frazzled) was first described as a tumor suppressor in colorectal cancer; however, the loss of expression in late-stage cancers led to studies regarding its potential role in metastasis. Correlative analyses in multiple tumor types show that DCC expression is lower in lymph node metastases, invading cells and disseminated cells [42-46]. Functionally, metastasis suppressor action has been defined using a Madin-Darby canine kidney cell model {40442} in which lymph node and lung metastases were impaired without inhibiting growth at the site of injection. There are two elements of this study which call into question the metastasis suppressor activity, however. First, MDCK are not a *bona fide* metastasis model in most laboratories. Second, the marker for tumor cells (i.e., luciferase) was significantly reduced despite overall tumor size being identical. This leaves open the possibility that a tumor suppressing effect was somehow masked by other cells that had been recruited to the site of tumor cell injection.

A mechanism of action for DCC is ambiguous because it has been implicated in diverse cellular functions, such as axon guidance. Among the mechanisms by which DCC is thought to direct cellular movement is by induction of the apoptosome [47]. Studies regarding induction of apoptosis in metastatic cells has not been measured to the best of our knowledge.

2.1.7 Caveolin-1—Caveolin-1 is the main component of caveolae, flask-shaped invaginations within the plasma membrane, and is also a scaffolding protein that links integrins to the tyrosine kinase FYN. Integrin stimulation and FYN activation leads to signaling through the Ras-ERK pathway, thereby promoting cell cycle progression [48,49]. Caveolin also interacts with EGFR, PDGFR-A and -B, endothelin, nitric oxide synthase, androgen receptor, and estrogen receptor- α [48,49]. Initially, caveolin-1 was described as a tumor suppressor; but recently, re-expression has been shown to reduce invasion, anchorage-dependent growth, angiogenesis and metastasis in some tumor models [48,50]. Many of the papers describing roles of caveolin-1 in metastasis-associated phenotypes did not present data showing lack of primary tumor growth inhibition; therefore, they have not yet met the strict definition of a metastasis suppressor. It is listed here because of textual assertions regarding orthotopic tumor growth. Although the mechanism by which caveolin-1 affects metastasis is unclear, it is presumed to be related to receiving signals from the local environment, since that is the role of caveolae.

2.2 Intracellular signaling molecules

After tumor cells have received signals from surrounding cells or matrices, they must interpret and transmit appropriate signals to alter tumor cell function. The transmission of this information is done by signaling molecules. Not surprisingly, the majority of metastasis suppressors identified to date are involved in signal transduction.

2.2.1 RKIP—Raf Kinase Inhibitor Protein was discovered as a metastasis suppressor gene in prostate cells [51,52]. Clinical data examining expression in primary tumors and matched lymph node metastases have confirmed a negative correlation between RKIP expression and tumor progression {38922}. RKIP directly binds Raf which, in turn, inhibits MEK1 activation with a concomitant decrease of downstream signaling. Since MEK and RKIP compete to bind to RAF, the presumptive mechanism of action is the regulation of ERK signaling, which has been shown to be pro-metastatic [53].

RKIP selectively regulates Raf1, but not Braf [54], suggesting that RKIP may exert an anti-metastatic effects only in certain cell types. In immune cells, RKIP has been implicated in NFκB and Snail signaling [55] which suggests that it might play a role in EMT. Another potential mechanism is that RKIP might regulate metastasis-regulatory microRNA (metastamiR, see below) [56].

2.2.2 Nm23—in 1988, Patricia Steeg discovered Nm23 (non-metastatic clone #23), the first metastasis suppressor to be identified [57]. Using differential colony hybridization to compare metastatic and non-metastatic variants of the murine K1735 melanoma cell lines, both pro-and anti-metastasis-associated molecules were identified [57]. Since it was the first metastasis suppressor discovered, Nm23 has been studied much more extensively than any of the others. With the exception of neuroblastoma, Nm23 expression is inversely correlated with poor survival and tumor grade for breast, gastric, ovarian, non-small cell lung, hepatocellular, and oral squamous cell carcinomas [58].

Transfection of Nm23 reduces motility in response to multiple growth factors in vitro [59-61]. EDG2 re-expression in Nm23-expressing cells restored motility in Nm23-H1-expressing cells while c-Met re-expression only partially restored motility, indicating that EDG2 regulation is closely associated with Nm23-induced metastasis suppression. The literature is replete with examples of Nm23 interactors, including Tiam1, Rad, glyceraldehyde 3-phosphate dehydrogenase, vimentin, various G-proteins and casein kinase 2. Notwithstanding the relatively “sticky” nature of this molecule and technical artifacts associated with many studies, the findings implicate Nm23 in an extensive array of cytoskeletal organizing and signaling pathways. Nm23 definitely interacts directly with and phosphorylates kinase suppressor of Ras (KSR), possibly altering KSR binding to other proteins and preventing downstream activation of the MAPK pathway. This hypothesis is strengthened by the observation that Nm23-H1 transfectants show reduced basal and stimulated MAPK phosphorylation [62].

Four distinct activities have also been reported for Nm23 — NDP kinase, RNA splicing, histidine kinase, exonuclease and maintenance of genomic stability [58]. It remains unclear which, if any, of these plays the critical role in suppressing cancer metastasis. NDP kinase- and exonuclease-disrupting mutants still suppress metastasis (to varying degrees), suggesting complex and overlapping roles in metastasis regulation.

2.2.3 JNKK1 / MKK7 / p38—Using a combination of MMCT and positional cloning, JNKK1/MKK4 (SEK1/MEK4/MAP2K4) were identified as a metastasis suppressor in prostate [63] and subsequently ovarian cancer [64]. Subsequent studies have shown that expression is lower in poor prognosis patients with pancreatic, breast and gastric cancers. However, clinical and experimental measurements of JNKK1 mRNA or protein expression have not always been consistent nor yielded similar conclusions. This is presumed to be because, like for so many signaling molecules, expression levels do not always signify activation.

In an ovarian cancer model, JNKK1/MKK4 activity arrested growth through the p38 arm of the SAPK signaling pathway [65]. Using a prostate cancer model, MKK7 (a specific activator of MKK4), but not MKK6 (a specific activator of p38), reduced overt metastases [66]. These elegant studies emphasize two critical points: (1) detailed exploration of branch points and signaling cascades must be measured; and (2) signaling cascades regulating metastasis are cell type-specific.

The careful, methodical and elegant studies performed by Carrie Rinker-Schaeffer and her colleagues deserves re-emphasis. Simplistic ‘-omic’ measurements can, and often do,

mislead. Moreover, the context of the cells and the stimuli impinging upon the tumor cell will alter the signaling cascades. Therefore, interpretation of all known variables and use of well-defined, specific reagents to measure the relevant activation states is essential.

2.2.4 DLC1—Deleted in liver cancer 1 (also known as Rho-GTPase activating protein 7 or START domain containing protein 12) was discovered in hepatocellular carcinoma [67]. Like RKIP and RhoGDI2, regulation of GTPase activity is presumed to be the mechanism by which DLC1 inhibits metastasis [68,69]. DLC1 also acts as a tumor suppressor, depending upon which cell line is used for the experiments [68,70,71].

DLC-1 gene expression is so commonly decreased in many human cancers (Durkin et al., 2007; Yuan et al., 2003) that its function is more likely as a tumor suppressor than as a metastasis suppressor. Yet, a recent report suggests that reduced expression in renal cell cancers correlates with increased invasion and poor prognosis (Zhang et al., 2009a). Thus, while DLC1 satisfies the definition of a metastasis suppressor in some cell lines and models, it does not in others.

2.2.5 RhoGDI2—RhoGDI2 is a member of a family of molecules that bind Rho GTPases, sequestering them in the cytosol and keeping them in the GDP-bound, inactive state. RhoGDI2 is a metastasis suppressor in bladder cancers [72] and Hodgkin's lymphoma [73] but promotes metastasis in other cancers [74,75]. In human bladder cancers, RhoGDI2 levels inversely correlate with development of metastatic disease. Remarkably, RhoGDI2 is an independent prognostic marker of recurrence following radical cystectomy [72]. Still, approximately one-third of patients with high levels of RhoGDI2 protein develop metastases, suggesting that other mechanisms might regulate the metastasis suppressor functions of RhoGDI2, such as phosphorylation, protein complex partners, protein turnover and subcellular localization.

RhoGDI2 has a relatively modest effect on RhoGTPase function; but, RhoGDI2 binds with Rac1, which can itself exert some anti-metastatic actions, presumably by altering cytoskeletal organization. When associations of RhoGDI2 and Src are accounted for, involvement of RhoGDI2 in Src signaling becomes an enticing possible mechanism of action [76]. Theodorescu and colleagues have proposed that specific phosphorylation of RhoGDI2 by Src may alter metastasis suppressor function by altering membrane-bound Rac1 [43793].

2.2.6 DRG1—Drg1 (also known as cap43/rit42/RTP/Ndrg1/TDD5) was originally discovered in colon carcinoma. Following DNA damage, Drg1 expression increases and the molecule translocates and accumulates in nuclei [77,78]{28462}. Like others of the more recently discovered metastasis suppressors, Drg1 has both tumor- and metastasis-suppressor activity depending upon the cell type in which it is introduced. Drg1 is a tumor suppressor in human bladder and pancreatic cancers [78]; but, over-expression in breast, colon, and prostate cancer cell lines suppressed metastasis without suppressing tumorigenicity [79]. In breast, prostate and liver cancers, expression is inversely correlated with progression [80] {38964}.

DRG-1 appears to function downstream of many cancer- and metastasis-associated signaling pathways, such as p53, PI3K/PTEN, PKC, hypoxia and some hormones. Re- or over-expression induces VEGF and IL8, which would suggest that the mechanism of action may involve regulation of angiogenesis. It has been speculated that the mechanism of action may be further associated with intravasation efficiency that results from changing vessel integrity.

2.2.7 SSeCKS/GRAVIN/AKAP12—The oncogene Src alters cell signaling and cytoskeletal organization by phosphorylating proteins that regulate transcription of tumor-promoting or tumor-suppressing genes. Src-suppressed protein Kinase C Substrate (SSeCKS) re-expression in rat prostatic cancer cells resulted in reduced lung metastasis in nude mouse models [81,82]. Expression in benign or well-differentiated prostate carcinomas, but not in highly aggressive and undifferentiated prostate lesions [82] is also indicative of metastasis suppressor activity.

SSeCKS is phosphorylated by protein kinase C (PKC) and is a scaffold for protein kinase A. Following stimulation by multiple growth factors or focal adhesion kinase, SSeCKS translocates and reorganizes the cytoskeleton. SSeCKS sequesters growth factors and binds F-actin when in its dephosphorylated state and, upon mitogenic signaling, it relinquishes actin binding and allows induction of signaling cascades leading to changes in the cytoskeleton. SSeCKS expression has also been correlated with decreased levels of multiple pro- and anti-angiogenic factors [81,82]. Recently, SSeCKS was shown to significantly decrease invasion and MMP-2 levels and suppress PKC-Raf/MEK/ERK pathway signaling induction [83]. In the latter study, podosome formation was inhibited independent of the actin cytoskeleton, but inhibition of MEK/ERK activation required actin cytoskeletal remodeling. Taken together, SSeCKS suppression of metastasis appears to involve multiple mechanisms that impact cell motility, invasion, and angiogenesis.

2.2.8 Gelsolin—Gelsolin is a Ca^{+2} -dependent actin binding protein. Cytoskeletal organization obviously plays a critical role in the ability of a cell to migrate. Gelsolin is a major actin-binding protein that has been shown to suppress metastasis in B16 murine melanoma cells [84]. Depending on the cell line and model system, gelsolin may play either tumor suppressive or metastasis suppressive roles [85]. Gelsolin expression has been recently correlated with metastatic potential in human colon adenocarcinoma cells [86].

2.2.9 HUNK—Hormonally Up-regulated Neu-associated Kinase (HUNK) was recently identified as a breast cancer metastasis suppressor by blocking actin polymerization leading to reduced cell motility [87]. HUNK can inactivate the actin-modulating protein cofilin-1 (CFL-1) by preventing its binding to protein phosphatase 2-A (PP2A) [87]. However, the lack of kinase activity was paradoxical. Therefore, additional studies will be required to identify specific context-dependent functions of HUNK in metastasis.

2.2.10 Ribonucleotide reductase M1—Ribonucleotide reductase M1 (RRM1) re-expression in the murine lung carcinoma cells suppress metastasis without blocking primary tumor growth [88]. RRM1 converts ribonucleotides to deoxyribonucleotides for DNA synthesis, suggesting that it may regulate dNTP pools which, in turn, would determine proliferative capacity. However, this hypothesis has not been tested. Interestingly, RRM1 up regulates PTEN and reduces FAK phosphorylation. A related ribonucleotide reductase, RRM2, has been studied with regard to cellular responsiveness to DNA damage and repair. Whether similar functions might alter tumor aggressiveness has been tested.

2.2.11 Caspase 8—Death receptor-activated apoptotic pathways function by activation of caspases. Activated caspase 8 cleaves caspase 3, leading to apoptosis. Using a neuroblastoma model, Stupack et al. re-expressed caspase and found significant suppression of metastasis [89]. Mysteriously, caspase 8 can promote cellular migration when apoptotic machinery is compromised [90]. In the latter case, caspase 8 was recruited to migration machinery following integrin ligation. While activity is not required for caspase 8-enhanced cell migration, association with FAK and calpain 2 was thought to be involved. Thus, caspase 8 exemplifies the differential roles that a single molecule may play in cellular behavior, depending upon other molecules with which it interacts.

Additionally, a role for caspase 8 in anoikis is intriguing. Anoikis is not exclusively the result of a failure of cells to adhere to a substrate. Anoikis can be induced if cells adhere to non-preferred substrates [91,92]. Thus, it is reasonable to hypothesize that analogous mechanisms might be involved in organotropism or failure to thrive following dissemination and arrest [93].

2.3 Extracellular Metastasis Suppressors

2.3.1 KISS1/kisspeptins—KISS1 was identified using subtractive hybridization between metastatic and nonmetastatic melanoma variants into which chromosome 6 have been introduced by MMCT [94,95]. Subsequently, it has been shown to suppress metastasis in multiple other tumor types [96-99]. Contrary to the expectation that the KISS1 gene would be on chromosome 6, it mapped to the long arm of chromosome 1. Using cDNA microarrays and chromosome 6 MMCT donors with defined deletions on the long-arm of chromosome 6, we found that KISS1 was regulated by TXNIP and CRSP3 (see below for details [100]).

KISS1 is transcribed as a 154 amino acid protein but rapidly processed into numerous polypeptides, termed kisspeptins [101,102]. An internal 54-amino acid polypeptide, termed kisspeptin (KP)-54 or metastatin, binds to KISS1R. Clinical reports generally support a positive correlation between KISS1 expression and metastasis-free survival [32].

Based upon the nature of the experimental studies showing KISS1 functionality as a metastasis suppressor when reexpressed in tumor cells, many of the metastatic cells suppressed by KISS1 re-expression surprisingly do not express KISS1R [33]. Thus, we hypothesized that there is a paracrine feedback loop in which KISS1/KP secreted by tumor cells acts upon stromal populations which, in turn, respond with growth inhibitory factors [32,103].

2.4 Regulators of transcription

2.4.1 CRSP3 and TXNIP—CRSP3 (also known as co-factor required for SP1 activity; DRIP130, Vitamin D regulatory interacting protein 130) and TXNIP (also known as thioredoxin interacting protein; TBP2; thioredoxin binding protein 2; VDUP, vitamin-D3 up-regulated protein) were identified as metastasis suppressors [100]. Both molecules were initially described by association with vitamin D and redox regulation. CRSP3, which maps to chromosome 6q23.2 induces TXNIP transcription, which, in turn, regulates the KISS1 metastasis suppressor. Thus, stimuli related to metabolism and/or signaling through vitamin D could affect metastasis, although this has not been tested directly.

2.4.2 BRMS1—Breast cancer metastasis suppressor 1 (BRMS1) was originally identified by analysis of differentially expressed genes in the metastatic breast carcinoma cell line MDA-MB-435 following MMCT of neomycin-tagged chromosome 11 [104]. Since the initial discovery in breast cancer, several labs have shown that BRMS1 suppresses metastasis of multiple tumor types including melanoma, ovarian, bladder and non-small cell lung carcinoma [105-108]. BRMS1 alters the expression of multiple metastasis-associated genes including osteopontin, urokinase plasminogen activator, fascin, epidermal growth factor receptor, CXCR4, as well as coordinately regulating metastamiR. Not surprisingly, given the wide range of genes affected by BRMS1 expression, BRMS1 affects multiple phenotypes implicated in cancer metastasis, gap junctional intercellular communication, migration, invasion, anoikis, and growth factor signaling.

The putative mechanism of action of BRMS1 is based primarily upon its interactions with multiple members of SIN3 histone deacetylation complexes. Expression arrays and proteomic analyses have revealed a complex pattern of differential gene expression when

BRMS1 is/is not expressed [109-112]. Analyses of putative downstream effectors has confirmed that osteopontin, phosphoinositide-4,5-bisphosphate and connexin 43 are *bona fide* effectors [113-116]. Clearly, each of these molecules is involved in cell-cell and cell-matrix signal transduction, reinforcing the notion that transcriptional activation of selected pathways is important in the regulation of the metastatic process.

2.4.3 LSD1—Lysine-Specific Demethylase 1 (LSD1) is an amine oxidase catalyzing the demethylation of histones and is a component of many chromatin remodeling complexes. More recently LSD1 was found to be an integral component of the Nucleosome Remodeling and Deacetylase (NuRD) protein complex, to inhibit invasion and to suppress metastasis in breast cancer models [117]. Interestingly, the NuRD complexes have also been implicated in promoting metastasis as several studies show important functions associated with histone deacetylation for the metastasis-associated proteins (MTA) [118-122]. Reminiscent of the complexes formed by BRMS1, the paradigm supported by LSD1 is that the function of chromatin remodeling complexes are clearly dependent on the specific composition of each complex.

2.4.4 KLF-17—Krupple-like factor 17 was identified in an unbiased screen using siRNA to knockdown molecules associated with mammary tumor metastasis [123]. Ectopic expression of KLF17 in metastatic 4T1 murine mammary carcinoma cells inhibited lung metastasis while knockdown in related 168FARN non-metastatic cells reduced EMT-related markers. KLF17 appears to bind the promoter region of the Id1 (inhibitor of differentiation 1) gene, inhibiting its transcription. If KLF17-expressing cells were injected intravenously, there was little effect on metastasis, suggesting that the step(s) of metastasis suppressed are early in the cascade. In clinical specimens, KLF17 expression was significantly lower in breast cancer specimens and, although the sample size was modest, appeared to be a potential biomarker for lymph node metastasis.

2.5 Post-transcriptional Regulators

It is readily apparent that metastasis is regulated by the differential expression of multiple genes that are regulated by signals impinging upon cells. However, recent data add further complexity to this situation. As alluded above, transcriptional regulation is only one of several key mechanisms that inhibit or promote metastasis. There are a number of proteins within a cell that are regulated post-transcriptionally.

2.5.1 metastamiR and Non-coding RNA—miRNA are small RNA genes are typically transcribed by RNA polymerase II to the pri-miRNA that adopts a characteristic hairpin loop structure. They are further processed to pre-miRNA by Drosha and exported to the cytoplasm where the enzyme Dicer processes the hairpin to a mature miRNA that associates with the RNA-induced silencing complex (RISC). Although the predominant mechanism of action is for the mature microRNA to bind to the 3'-UTR of mRNA to prevent protein translation, emerging evidence suggests that they may also bind to promoter regions of genes to regulate expression and stabilize messenger RNAs that can be translated more rapidly in periods of stress [124,125].

miRNA have been reported to play key roles in cancer, recurrence, development of metastases and survival (reviewed in [126-129]). The first suppressing metastamiR was identified by Tavazoie et al., who compared miRNA expression in metastatic variants derived from the human breast carcinoma cell line, MDA-MB-231 [130]. Of the six miRNA identified in their studies, to, miR-335, and -206, suppressed metastasis in vivo without preventing tumorigenesis. Both miR-335 and -206 inhibit migration and invasion in vitro. miR-335 targets SOX4 (SRY-box containing transcription factor), PTPRN2 (receptor type

tyrosine protein phosphatase), MERTK (c-Mer tyrosine kinase), and possibly TNC (tenascin C). Importantly, each miRNA impacts several downstream pathways.

miR-146a and -146b genes are encoded on different chromosomes but their mature sequence are predicted to overlap. Both miR-146a and -146b inhibit invasion and migration in vitro, prompting us to test whether they also suppressed metastasis, which they do [131,132]. Interestingly, miR-146a and -146b suppressed metastasis that may involve targeting of EGFR [132] or ROCK1 [128], both of which are involved in promoting invasion and metastasis. In clinical samples, miR-146a expression is inversely correlated with prostate cancer progression, further supporting a metastasis suppressor function for this metastamiR. While inhibition of any step in the metastasis cascade precludes metastasis, each metastamiR could suppress metastasis by simultaneously (?) targeting multiple steps.

MetastamiR can also promote metastasis. miR-10b was discovered by Ma and colleagues [133]. Having hypothesized that specific miRNA could regulate tumor progression, she found that miR-10b was highly expressed only in metastatic breast cancer cell lines compared to primary human mammary epithelial or spontaneously immortalized cells. She then went on to demonstrate that miR-10b enhanced migration and invasion in vitro and metastasis in vivo. Importantly, she and her colleagues identified a pathway where the pro-metastatic gene TWIST1 up-regulates miR-10b that targets HOXD10 leading to an increase in RHOC. In recent months, the same group demonstrated that antagonists to the microRNA can be used effectively in preclinical models [134]. The timing of administration of the antagonists has revealed key steps in the metastatic cascade in which miR-10b is operating.

While metastamiR have only been recognized for slightly more than two years, the rate of discovery of this important family of molecules is impressive. MetastamiR are components of complex pathways and are often expressed downstream of pro- or anti-metastatic signals, including pathways regulated by NF κ B, EGFR, TWIST1, BRMS1, ZEB1/2 and HIF1 α (reviewed in [1]).

Non-coding RNA involved in metastasis are not limited to miRNA. Recently, the large intergenic non-coding RNA (lincRNA) HOTAIR was found to promote metastasis [135]. HOTAIR associates with the chromatin remodeling complexes Polycomb Repressive Complex 2 (PRC2) and LSD1 and alters epigenetic marks on histones which, in turn, alter gene expression. Undoubtedly, the number of other non-coding RNA implicated in metastasis will grow.

3 Conclusions and Perspectives

The mere fact that metastasis suppressors allow growth at orthotopic sites, but prohibit growth at ectopic sites is clear evidence that the microenvironment is an important contributor to the metastatic cascade. To extrapolate that there are genes within cells that are differentially regulated by the microenvironment is not a stretch. The data presented above, compiled from numerous papers, demonstrate that metastasis suppressors can be proximal or distal mediators of signals from the microenvironments in which tumor cells find themselves.

Some of the metastasis suppressors promote homotypic cell-cell adhesion (e.g., E.-cadherin) which, in turn, slows initial migration away from the primary tumor mass. A large number of metastasis suppressors (e.g., Nm23, tissue inhibitors of metalloproteinases (TIMP), SseCKS) prohibit motility and/or invasion so that tumor cells are more likely to remain at the primary tumor. Still other metastasis suppressors reduce survivability of cells during transit from the primary tumor to secondary sites (e.g., caspase-8, BRMS1, KAI1.) And

finally, a subset of the metastasis suppressors prohibit growth of tumor cells after they have already disseminated (e.g., KISS1, MKK4, p38, MKK7).

Tumor cells do not determine when nor where metastases develop. Regardless of the step in the metastatic cascade, tumor cells are impinged by growth promoting and growth inhibitory signals. Depending upon how they respond, they will die, remain quiescent or proliferate. It is only when they grow at secondary sites that they become *bona fide* metastases. Although not yet discovered, we predict that a subset of metastasis suppressors will eventually be identified that explain the organotropism first described by Stephen Paget.

It has long been recognized that metastasis is complicated. Advances in our understanding of this deadly process have been slowed because of its complexity. As we gain a greater foothold into the inherent genetic changes in cells, it is becoming increasingly apparent that the interplay between cancer-associated genes and the environment must play a greater role in our modeling and conceptualization of the mechanisms involved in cancer metastasis. Our hope is that the presentation of the facts known to date can stimulate other laboratories to take up the challenge.

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Table 1
Metastasis suppressors and proposed mechanisms

Metastasis suppressor	Proposed Mechanism(s) of Action	Step(s) in Metastasis implicated
BRMS1	Transcriptional regulation via interaction with SIN3:HDAC complexes; down-regulates PtdIns(4,5)P ₂	Invasion (Intravasation/Extravasation) Motility Adhesion Colonization
Caspase 8	Induction of apoptosis if cells bind to un-liganded integrins	Transport Survival in circulation Adhesion
E-cadherin	Cell: cell interactions	Local invasion Intravasation
N-cadherin	Cell: cell interactions	Local invasion Intravasation
Cadherin-11	Cell: cell, cell: matrix interactions	Local invasion Intravasation Motility
Caveolin-1	Altering caveolae function	Invasion (Intravasation/Extravasation) Possibly colonization
CD44	Hyaluronic acid receptor; osteopontin receptor stem cell marker (selected)	Adhesion Invasion Possibly colonization
DCC	Regulates cytoskeletal organization; regulates MAPK signaling	Motility Invasion (Intravasation/Extravasation) Survival in circulation
DLC1	Rho-GTPase activating protein; regulates cytoskeletal structure	Motility Invasion (Intravasation/Extravasation) Survival in circulation
DRG1	Unknown	Angiogenesis Possibly colonization
Gelsolin	Regulates cytoskeletal structure; reduces motility	Motility Adhesion Invasion (Intravasation/Extravasation)
HUNK	Protein kinase	Motility Invasion (Intravasation/Extravasation)
KAI1	Interacts with endothelial DARC to induce apoptosis	Intravasation Survival in circulation
KISS1 (kisspeptins)	Maintains dormancy at secondary sites	Colonization
KISS1R	G-protein coupled receptor	Colonization
KLF17	Transcription	Invasion (Intravasation/Extravasation) Local Invasion
LSD1	Chromatin remodeling	Motility Invasion (Intravasation/Extravasation) Possibly colonization
MKK4	Stress-Activated MAPK signaling	Colonization
MKK7	Stress Activated MAPK signaling	Colonization
p38	Stress Activated MAPK signaling	Colonization
Nm23	Phosphorylates KSR to prevent downstream activation of MAPK pathways	Motility Invasion (Intravasation/Extravasation) Colonization
OGR1	GPCR signaling	Invasion (Intravasation/Extravasation) Migration Possibly colonization
RhoGDI2	Regulates Rho; negatively alters endothelin 1 and neuromedin U expression	Adhesion Invasion (Intravasation/Extravasation)

Metastasis suppressor	Proposed Mechanism(s) of Action	Step(s) in Metastasis implicated
RKIP	Competitive inhibitor of RAF1-MEK interactions	Survival in circulation Possibly colonization Motility Invasion (Intravasation/Extravasation) Adhesion
RRM1	Increases PTEN expression; decreases FAK phosphorylation	Motility Invasion (Intravasation/Extravasation) Adhesion
SSeCKS	Scaffold protein for PKA and PKC; inhibits osteopontin, VEGF expression; up regulates vasostatin	Angiogenesis Adhesion Migration Invasion (Intravasation/Extravasation)
TIMPs	Inhibit metalloproteinases; signaling	Local invasion Invasion (Intravasation/Extravasation) Adhesion Colonization Angiogenesis

Adapted from Hurst and Welch [1]