PATHOBIOLOGY IN FOCUS

Metastasis suppressors: functional pathways

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Metastasis is a complex process and a major contributor of death in cancer patients. Metastasis suppressor genes are identified by their ability to inhibit metastasis at a secondary site without affecting the growth of primary tumor. In this review, we have conducted a survey of the metastasis suppressor literature to identify common downstream pathways. The metastasis suppressor genes mechanistically target MAPK, G-protein-coupled receptor, cell adhesion, cytoskeletal, transcriptional regulatory, and metastasis susceptibility pathways. The majority of the metastasis suppressor genes are functionally multifactorial, inhibiting metastasis at multiple points in the cascade, and many operate in a context-dependent fashion. A greater understanding of common pathways/molecules targeted by metastasis suppressor could improve metastasis treatment strategies.

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INTRODUCTION

Despite advances in diagnosis, surgery, radio- and chemotherapy, a strong need remains for improvements in metastasis-related cancer mortality.^{1,2} Most therapies address fundamental oncogenic events that are present in a metastasis, but an alternative is to address the metastatic process itself.

Clinical and translational advances pertaining to the metastatic process may be thwarted by our poor understanding of the molecular and biochemical mechanisms involved in the process of metastasis. Metastasis is a complex multistep sequential process involving invasion of cancer cells, intravasation, circulation, arrest in the capillary bed of secondary organ, extravasation, and colonization at the secondary site. Apart from being complex, metastasis is also highly inefficient. Experimental studies have shown that, among all the cancer cells injected into the circulation, very few (~0.01%) have the capacity to successfully metastasize to distant organ.^{3,4} This inefficiency in metastatic ability has been attributed to crucial rate-limiting steps of metastasis namely the host immune system in the circulation, extravasation, and post-extravasation survival in colonization.^{4–7} Cells that metastasize to secondary sites may not necessarily proliferate immediately and could remain dormant for a long period, until the microenvironment at secondary site becomes congenial for its proliferation.^{8,9}

Characterization of pathways/genes/proteins that specifically affect metastasis, metastasis suppressor or metastasis promoters, would provide insights into the molecular mechanisms of metastasis and could launch clinical-

translational initiatives. This review will focus on the metastasis suppressor gene field, exploring the mechanistic pathways involved and suggested translational sequelae.

METASTASIS SUPPRESSORS AND THEIR FUNCTIONAL IDENTIFICATION

Like tumorigenesis, both stimulatory and inhibitory pathways regulate metastasis. Some inhibitory pathways are pertinent to both tumor growth and spread; the metastasis suppressor gene field has limited itself to those genes that are specific to the metastatic process. Metastasis suppressors are a class of genes which, when overexpressed (or re-expressed), cause inhibition of cancer metastasis to distant organs without affecting the size of the primary tumor. Because no *in vitro* assays exist for metastasis, credentialing of a new suppressor necessarily involves *in vivo* experiments. The gold standard is spontaneous metastasis assays, where a primary tumor forms and seeds out metastases; experimental (eg hematogenous) metastasis assays have been used in concert with separate primary tumor studies.

Metastasis suppressor genes have been identified in many ways, but often show reduced expression in highly metastatic cells compared to poorly or non-metastatic tumorigenic cells.¹⁰ The first metastasis suppressor gene, NM23 (NME), was identified by its overexpression in murine K-1735 melanoma lines with poor metastatic potential as compared with related lines of high metastatic capacity.¹¹ Several techniques including microcell-mediated chromosome transfer, differential display, subtractive hybridization,

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microarrays, RNAseq and serial analysis of gene expression have been employed to identify genes or chromosomal regions which suppress metastasis.¹² In addition to genes, multiple types of RNAs have been demonstrated to have metastasis suppressor functions.

Metastasis suppressors can act by inhibiting one or more steps in the metastatic cascade and regulate a wide range of biochemical signaling pathways.¹³ Most have been found to be active in several biochemical pathways, suggesting that exerting multiple control points on a genomically unstable cell population may be advantageous. Apart from inhibiting metastasis, metastasis suppressors may have a 'normal' function in the induction or maintenance of development and differentiation pathways.^{14–17} Examples include Raf kinase inhibitory protein (RKIP) (PEBP), which controls pulmonary airway tube formation in *Drosophila*, and brain, pancreas and retinal development in the mouse.^{18,19} NM23 controls the differentiation of presumptive adult tissue in the imaginal discs of *Drosophila*, and the knockout mouse has a mammary differentiation defect preventing nursing.^{20–22} The MKK4 (mitogen-activated protein kinase kinase 4) suppressor gene, as in mammalian cells, controls P38 and JNK-based responses to cell stress in *Drosophila* development, altering mitosis and motility.²³ In contrast to their function in development and differentiation, where pathways are turned on and off in a controlled manner, these pathways operate in the instability of cancer cells as metastasis suppressors.

PATHWAYS TARGETED BY METASTASIS SUPPRESSOR GENES

We have categorized the metastasis suppressors genes into different pathways based on their predominant known mode of action. As will become apparent, few have only one biochemical function. An overview of metastasis suppressor pathways is listed in Table 1. A literature search reveals that the mitogen-activated protein kinase (MAPK) and cytoskeletal signaling pathways are the most common activities of metastasis suppressors: Whether that means that these pathways predominate biologically, or are just the best studied, remains an open question. Certainly, control of gene expression and other pathways may be as important, but less studied and more intricate.

MAPK Pathway

This signaling cascade starts with the binding of an extracellular mitogenic ligand to the membrane receptor (ie EGFR, PDGFR) leading to activation of Ras (a GTPase) which activates series of MAPKs via MAP3K, MAP2K, MAPK and finally activation of transcription factors (Figure 1). In simplistic terms, the ERK subpathway is thought to regulate mitosis, while the JNK and P38 subpathways regulate stress responses. The ERK–MAPK signaling plays a crucial role in cancer and metastasis. Studies on breast carcinomas, and head and neck squamous-cell carcinoma have reported a positive

correlation between activated ERK expression, metastases and patient survival.²⁴ Similarly, in studies using ERK inhibitors (PD 098059) or site-directed mutagenesis, ERK activation was functionally associated with *in vivo* metastasis,^{25,26} *in vitro* motility and invasion^{27,28} and the epithelial–mesenchymal transition (EMT).²⁹ Metastasis suppressors with known ties to the ERK pathway are listed below.

NM23 (NME/NM23H1)

NM23 was the first metastasis suppressor identified, which showed reduced expression (by differential hybridization) in highly metastatic compared to poorly metastatic K-1735 murine melanoma cell lines. As a metastasis suppressor, its enforced expression suppressed metastasis without altering tumor growth in variety of cancer cell lines of different origins.³⁰ Like its *in vivo* metastasis-inhibitory phenotype, overexpression of NM23 in several carcinoma cells (breast, melanoma, etc) reduced *in vitro* motility of cells to numerous chemoattractants and inhibited colony formation in soft agar, without affecting the cellular proliferation.³¹ In humans NM23/NDPK family consists of 10 members (NM23H1–H10) including NM23H1 and NM23H2 which share 88% homology.³² Like NM23H1, a variety of metastasis model systems have demonstrated NM23H2 as a metastasis suppressor gene and its expression levels inversely correlate with metastatic potential.^{33–36} NM23 overexpression in MDA-MB-435 breast carcinoma cells reduced ERK–MAPK activation levels compared to vector control.^{37,38}

Biochemically, NM23 is known to have nucleoside diphosphate kinase (NDPK) activity,^{16,39–41} Histidine protein kinase activity (HPK)^{42,43} and a 5'–3' exonuclease activity.⁴⁴ As an NDPK, it catalyzes the transphosphorylation of the γ -phosphate of a nucleoside triphosphate to a nucleoside diphosphate with a phosphorylated-histidine (H118) intermediate. As an HPK, the same phosphorylated-histidine 118 NM23 intermediate transfers a phosphate to a protein substrate, a histidine in all NME2 reported pathways but a histidine or serine in some NME1 pathways. Developmental studies in *Drosophila* demonstrated that the NDPK activity was necessary but not sufficient for normal differentiation, and roles for subcellular provision of nucleotides have been postulated.⁴⁵ The motility-suppressive function of NM23 is well correlated with its HPK activity in site-directed mutagenesis experiments.^{46,47} The physiological substrates for HPK activity potentially leading to NM23-mediated metastasis suppression are incompletely known. Among several known HPK substrates of NM23, phosphorylation of kinase suppressor of ras (KSR) by NM23 is known to inhibit ERK–MAPK pathway (Figure 1).^{37,38,48} In this study, NM23 phosphorylated KSR-serine (392) and altered its scaffold function, possibly by altering the docking of proteins and/or by altering KSR intracellular localization, leading to reduced ERK activation. Independent publications have reported a protein–protein interaction of NM23 and KSR affecting *Caenorhabditis elegans* development,⁴⁹ and a functional

Table 1 A list of bona fide metastasis suppressor genes

Pathways targeted	Genes	Tumor type/site	Mech. of action	Type of metastasis
MAPK pathway	NM23 ³⁷	Breast	1-Phosphorylation of KSR leading to reduced ERK1/2 activation	Reduces both experimental & spontaneous metastases
		Melanoma ^{163–165}	2-LPA signaling ⁵⁴	
		Colon ^{166,167}		
		Oral ^{36,168}		
	RKIP ⁶⁰	Prostate	Inhibits RAF mediated MAP/ERK kinase phosphorylation	Reduces spontaneous metastases
	MKK4 ^{66,70,71,72}	Prostate, ovarian cancer	MAP2K activates p38 and/or JNK	Reduces spontaneous metastases
	MKK6 ⁶⁶	Ovarian cancer	Activates MAPK p38	Reduces experimental metastases
	MKK7 ⁷²	Prostate cancer	Activates MAPK JNK	Reduces spontaneous metastases
Adhesion proteins	KAI1 ^{169–171}	Prostate	Cell adhesion and Integrin signaling	Reduces spontaneous metastases
	CD44 ^{89,172,173,174,175}	Prostate	Affects cellular adhesion by hyaluronic acid and osteopontin signaling	Reduces spontaneous metastasis
	E-cadherin ^{176,177,178,179}	Colon, pancreatic and ovarian cancer	Ca ²⁺ -dependent cell adhesion	Reduces spontaneous metastases
Cytoskeletal signaling	Gelsolin ¹⁰⁶	Melanoma	Actin-regulatory protein helping in actin polymerization	Reduces spontaneous metastasis
	Rho–Rac pathway: RhoGDI2 ¹⁰⁹	Bladder cancer	Rho–Rac signaling	Reduces experimental metastasis
	DLC-1 ¹¹¹	Breast cancer	Rho GTPase activating protein which affects cytoskeletal remodeling	Reduces spontaneous metastasis
G-protein-coupled receptors pathway	KISS1 ^{114,115}	Melanoma	Cytoskeletal reorganization	Reduces both experimental and spontaneous metastases
		Breast ¹¹⁶		
		Lungs		
	Protein kinase A/C pathway: SSeCKS/Gravin/AKAP12 ^{120,121,180}	Prostate cancer	PKA-mediated activation of Src kinase	Reduces spontaneous metastasis
Apoptotic pathway	Caspase-8 ^{123,181,182}	Neuroblastoma	Increases integrin-mediated cell death	Reduces spontaneous metastasis

Table 1 Continued

Pathways targeted	Genes	Tumor type/site	Mech. of action	Type of metastasis
Transcriptional complexes	GAS1 ^{124,183}	Melanoma	Induces apoptosis through caspases 3 and 9	Reduces spontaneous metastasis
	BRMS1 ^{184,185}	Melanoma, ovarian cancer	Chromatin remodeling (polyubiquitinated p300, a histone acetyltransferase) and PI3K signaling	Reduces spontaneous metastasis
Anti-metastatic miRNAs (Metastamirs)	MYC ¹⁴¹	Breast cancer	Transcriptional silencing of α v and β 3 integrin subunits	Reduces spontaneous metastasis
	miR-146a/b ^{147,186}	Breast cancer	Reduces NF κ B and EGFR pathway	Reduces experimental metastasis
Long non-coding RNA (lncRNA)	miR-206	Breast cancer	Target transcription factor SOX4 and extracellular matrix component tenascin C	Reduces experimental metastasis
	miR-335 ¹⁴⁸	Breast cancer	Binds to NF90 and causes its degradation	Reduces experimental metastasis
Metastasis susceptibility genes	LncRNA LET ^{153,154}	Hepatocellular carcinoma and colon carcinoma		
	NKILA (NF κ B-activating lncRNA) ^{155,156}	Breast cancer	Negative regulator of NF κ B signaling	Reduces experimental metastasis
	Cadm1 ¹⁶⁰	Breast cancer	Sensitizes tumor cells to immune-surveillance mechanisms by CD8+ T cells	Reduces spontaneous metastasis

NM23–KSR interaction regulating ERK activation was reported in HEK293 cancer cells.⁵⁰ Similarly, tumor cells expressing an NM23 histidine-kinase-deficient mutant (P96S) showed high levels of ERK activation comparable to vector control.³⁸ Therefore, it is very likely that differential expression of NM23 in metastatic vs non-metastatic tumor cells might impact ERK activation via KSR scaffold protein which could, at least in part, mediate NM23 actions.^{46,51}

The mechanism of NM23 action leading to metastasis suppression is likely to be multifactorial. Apart from activities described above, NM23 binding proteins, which titer out 'free NM23' or reduce the titer of metastasis-promoting proteins (such as EBV), could lead to inhibition of its ability to suppress metastasis;^{52,53} altered gene expression of downstream targets (lysophosphatidic acid receptor LPA1/EDG2)⁵⁴ is potential candidates which has shown promising results for mediating NM23 activities, and recent observations on endocytic trafficking are likely contributory.^{20,21,45}

Raf kinase inhibitory protein

RKIP binds Raf-1 and is an inhibitor of Raf-stimulated ERK–MAPK signaling (Figure 1);⁵⁵ it is also a member of the phosphoethanolamine-binding protein (PEBP) family. RKIP inhibition is isoform specific and only works on Raf-1, and not on B-Raf.⁵⁶ RKIP was identified as a metastasis suppressor in a screen comparing metastatic prostate cancer cell lines to non-metastatic cells.⁵⁷ It has been proven as functional metastasis suppressor in multiple solid tumor types such as prostate and triple-negative breast cancers.^{57,58} Generally, RKIP expression is lost in poor-prognosis tumors.⁵⁹ Studies using both *in vitro* and *in vivo* breast cancer models have demonstrated that expression of RKIP blocks multiple steps of the metastasis including angiogenesis, local invasion, intravasation, and colonization.^{57,60} Enforced expression of RKIP reduced the invasive potential of breast cancer cells without affecting their growth.⁵⁷ Similarly, in a xenograft model its overexpression blocked intravasation, extravasation as well as colonization of tumor cells.

In addition to its RAF inhibitory action, RKIP has other biochemical activities which contribute to metastasis suppression. These include binding to and inhibition of signal transducer and activator of transcription 3 (STAT3),⁶¹ binding to G-protein-coupled receptor kinase (GRK2),⁶² MDA-9/Syntenin,⁶³ centrosomes and kinetochores.⁶⁴

These examples bring up a remaining question of the interaction between an antiproliferative pathway for ERK and a metastasis pathway independent of primary tumor growth. Multiple studies have indicated that tumor growth in a primary site is not necessarily the same as growth in a distant site,⁶⁵ suggesting that context-dependent activation of a proliferative pathway may be key. Alternatively, 'other' functions of these suppressors may impact colonization by distinct pathways. Colonization is certainly more than just proliferation, involving stem cell function, viability pathways, stress responses, and immune evasion for example.

Figure 1 Mitogen-activated protein kinases (MAPK) pathway highlighting metastasis suppressor targeting sites.

The stress activated protein kinase (SAPK) portion of the MAPK pathway terminates in P38 and JNK activation. The SAPK signaling pathways are generally activated by a variety of environmental stresses including pH changes, UV irradiation, hypoxia, cytokines or growth factor deprivation and treatment with chemotherapeutic agents.⁶⁶ Intuitively, it is easy to envision a tumor cell arriving in a foreign tissue as being stressed, and lack of an adaptive response would be detrimental to colonization. P38 has also been functionally implicated in metastatic dormancy, which is a form of metastasis suppression.^{67–69} Other contributions of the SAPK, for instance in immune regulation, may also be functionally important.

Mitogen-activated protein kinase kinase 4

MKK4 is a member of the MAP kinase kinase family which directly phosphorylates and activates JNK or P38 in the SAPK. Human prostate cancer primary tumors with higher Gleason grade, and metastatic ovarian cancer patients, showed reduced expression of MKK4. MKK4 acts as a functional metastasis suppressor wherein its ectopic expression in highly metastatic cells suppressed metastasis significantly (80–90%) in both ovarian and prostate cancer cells.^{70,71} Interestingly, it was observed that for suppressing metastasis in ovarian cancer models, MKK4 signaled through the p38 arm,⁶⁶ whereas in prostate cancer models, MKK4 signaled through the JNK arm.⁷²

Mitogen-activated protein kinase kinase 6/7

Mitogen-activated protein kinase kinase 6/7 (MKK 6 and 7) play a central role in the SAPK signaling pathway. MKKs 6 and 7 occupy slightly different positions in the MAPK cascade: In response to cellular stress generally MAPKKK is activated which can, in turn, activate MKK4, MKK7, or MKK3/MKK6. Activation of MKK3, MKK6, and/or MKK4 (but not of MKK7) can lead to phosphorylation of all the four members of the mammalian p38 MAPK family members (p38 α , p38 β , p38 γ , and p38 δ).⁷³ Activation of p38 in metastatic cells is required for its survival in dormant state at the secondary site.^{74,75} It is speculated that activation of p38-induced dormancy resulted in an unfolded protein response, which is an adaptive pathway that might help the survival of dormant tumor cells at the secondary site.⁶⁷ However, depending on the model system and the isoform involved, MKK data show inconsistencies in literature. In an ovarian cancer model, MKK6 overexpression suppressed metastatic colonization whereas MKK7 had no effect. However, in prostate cancer ectopic expression of the MKK7 (JNK-specific kinase) suppresses formation of overt metastases, whereas the p38-specific kinase MKK6 had no effect.^{66,72,76} The inconsistency of MKK4, 6, 7 effects in the various models may indicate the importance of the pathway, but redundant mechanisms for its traversal. Alternatively, do the MKK proteins serve other, non-MAPK functions? It is noteworthy, however, that the SAPK pathway has been widely reported as an oncogene,^{77,78} and that its function may be context dependent.

Adhesion Proteins

Adhesion proteins modulate cell:cell and cell:extracellular matrix interactions, with resultant effects on proliferation, survival, motility, immune function, and other aspects of metastasis. Multiple adhesion molecules have been credentialled as metastasis suppressors.

KAI1

KAI1/CD82 is a member of tetraspanin family, which is characterized by its four transmembrane and two extracellular domains. It was first identified in T-cell activation study;⁷⁹ however, its role as a metastasis suppressor was highlighted later in a somatic cell hybridization of highly metastatic and non-metastatic rat prostate cancer cells.⁸⁰ Decreased expression of KAI1 has been widely correlated with an aggressive cancer phenotype in several cancer types, including pancreatic, hepatocellular, bladder, breast, and non-small cell lung cancers.^{81–83} Similarly, in breast cancer cells its overexpression led to poor colonization in soft agar, reduced invasion, and significantly suppressed both spontaneous and experimental metastasis.⁸⁴ KAI functions as an adaptor protein that forms oligomeric complexes with its binding partners, including integrins, EGFR, GTPases, and other cell adhesion molecules. Since the MAPK pathway lies downstream of some of these targets, it may be functionally

involved. KAI1-mediated metastasis suppression has also been linked to abrogation of EGFR and integrin signaling, possibly by increasing their endocytosis.⁸⁵ This hypothesis is also supported by the presence of internalization consensus sequence in KAI1, which is unique in the tetraspanin family.⁸⁶

CD44

CD44 is a transmembrane glycoprotein involved in cell–cell and cell–matrix interactions and acts as a receptor for the extracellular matrix components hyaluronic acid⁸⁷ and osteopontin.⁸⁸ CD44, like a metastasis suppressor gene, has downregulated mRNA and protein expression in highly metastatic prostate cancer.⁸⁹ Similarly, overexpression of CD44 (Mr 85 000, standard form) in highly metastatic AT3.1 rat prostatic cells suppressed their metastatic ability to the lungs without affecting their *in vivo* growth rate or tumorigenicity.⁸⁹ CD44 exists in multiple isoforms, some of which have been demonstrated to increase metastasis.⁹⁰ Switching of CD44 isoforms through cancer progression has been reported.⁹¹ The range of CD44 isoforms and their contributions to metastasis remain incompletely understood. The role(s) of CD44 in metastasis suppression are likely multifactorial and can involve immune function,⁹² interactions with receptor tyrosine kinase signaling,⁹³ cytoskeletal changes,^{94,95} particularly in response to hyaluronic acid, stem cell function, TGF- β function,⁹⁶ etc. The CD44–hyaluronic acid pathway is the subject of multiple translational initiatives.

E-Cadherin

Cadherins are transmembrane glycoproteins that form Ca⁺² dependent homotypic complexes and mediate cell–cell interactions mainly in epithelial cells. There are 20 members of cadherin family, which show tissue-specific expression patterns.⁹⁷ Loss of E-cadherin in a variety of tumor cell types is associated with the EMT and correlates with increased invasion and metastasis.⁹⁸ Re-expression of E-cadherin in highly invasive epithelial tumors suppressed the invasive potential of these cells.⁹⁹ Similarly, high E-cadherin expression in primary tumor inhibits shedding of tumor cells from the primary tumor,^{100,101} highlighting a metastasis suppressor role. E-cadherin modulation of metastasis has been reported,^{98,102,103} with the caveat that tumor suppressive roles have also been reported;¹⁰⁴ metastasis modulation may involve adhesive or other signaling events.

Cytoskeletal Signaling

Cellular migration and other cellular deformability events in response to microenvironmental cues are guided by a dynamic cytoskeleton. Multiple proteins in traditional motility/invasion signaling pathways have been credentialed as metastasis suppressors, affecting the mechanics of ratcheting a cell forward. Less clear in the literature is the contribution of cytoskeletal pathways to growth in a distant site, metastatic colonization. Cytoskeletal pathways may affect viability (such as the anoikis process), immune intravasation,

mechanical signaling from environmental stiffness, and other aspects which need further study. These end points may be as useful as motility in translating this field toward clinical application. Multiple molecules acting at different level of cytoskeletal signaling have been credentialed as metastasis suppressors.

Gelsolin

Gelsolin is an actin-binding protein that regulates cell motility by severing actin. Gelsolin also regulates cell morphology, proliferation, and apoptosis. Studies on Gelsolin expression have highlighted its reduced expression in several cancer types, including breast, colon, ovary, and prostate cancer. Overexpression and knockdown studies on Gelsolin have highlighted its role in inhibiting the EMT in breast cancer,¹⁰⁵ and as a suppressor of metastasis in B16 melanoma cells.¹⁰⁶ Gelsolin functions as a switch that controls E- and N-cadherin conversion, that is, knockdown of gelsolin causes a reduction in E-cadherin and enhanced expression of N-cadherin via a transcription factor, Snail.

RhoGDI

The Rho/Rac proteins constitute a subgroup of the Ras superfamily of small GTP hydrolases which were originally implicated in the control of cytoskeletal events. However, it is also known to regulate diverse cellular functions, including cell polarity, vesicular trafficking, cell cycle, transcription, and proliferation.¹⁰⁷ Upon activation, it switches from an inactive GDP-bound form to an active GTP-bound form which can interact with a plethora of different effectors mediating its different cellular functions.¹⁰⁷ Rho GTPases are known to contribute to most steps of cancer initiation and progression, including proliferation potential, survival and evasion from apoptosis, angiogenesis, tissue invasion, and the establishment of metastases. RhoGDI stabilizes the GDP-bound form of Rho and sequesters it in an inactive, non-membrane localized, cytoplasmic compartment, inactivating Rho–Rac signaling.¹⁰⁸ RhoGDI2 expression was diminished as a function of primary tumor stage and grade in human bladder and in several other cancers.¹⁰⁹ Enforced expression of RhoGDI2, into cells having reduced expression with metastatic ability, suppressed experimental lung metastasis without affecting *in vitro* growth, colony formation, and *in vivo* tumorigenic potential.¹⁰⁹ RhoGDI2 also affects gene expression by an incompletely characterized mechanism, which may also contribute to metastasis suppression.¹¹⁰

DLC-1

When overexpressed in sublines of the MDA-MB-435 breast carcinoma, DLC-1 resulted in reduced migration, invasion, and significantly suppressed pulmonary metastases.¹¹¹ DLC-1 is a GTPase activating proteins (GAP) important for switching the active GTP-bound state to the inactive GDP-bound state of Rho proteins.¹¹² A caveat is that, DLC-1 is also known

to have potential tumor suppressor activity in hepatocellular carcinoma.¹¹³

G-Protein-Coupled Receptors

G-protein-coupled receptors (GPCRs) constitute a large class of druggable signaling proteins with a plethora of downstream activities. GPCR family members contains a typical seven transmembrane α -helical region. They detect many extracellular signals and transduce them to heterotrimeric G proteins, which further carry these signals to intracellular effectors (cAMP, PKA, PI3K, etc), and thereby play an important role in various cellular responses.

KISS1

KISS1 was identified as a metastasis suppressor gene by subtractive hybridization on metastatic melanoma cells (C8161) with and without human chromosome 6.^{114,115} Overexpression of KISS1 inhibited metastases formed by breast and melanoma cells, without altering the size of primary tumors.¹¹⁶ The mechanism of action of KISS1 took an unexpected turn when it was reported to encode a secreted neuropeptide (designated as Metastatin and Kisspeptin) for an orphan G-protein-coupled receptor (hGPR54).^{117,118} Melanoma cells overexpressing hGPR54 developed reduced metastases upon treatment with Metastatin, while in *in vitro* assays it inhibited chemotaxis and invasion. These ligands (Metastatin, Kisspeptin) stimulate PIP₂ hydrolysis, Ca²⁺ mobilization, arachidonic acid release, ERK1/2 and p38 MAP kinase phosphorylation and stress fiber formation, leading to reduced cellular proliferation.¹¹⁸ In another study, constitutive upregulation of KISS1 in HT10810 cells resulted in decreased nuclear factor- κ B (NF κ B) activation, which in turn led to reduced MMP9 transcription and resultant changes in invasion.

Src-suppressed C kinase substrate

Src-suppressed C kinase substrate (SSECKS) is a scaffolding protein for many important signaling intermediates. It has metastasis suppressive activity (complicated by reports of tumor suppressive activity). In studies of motility, loss of SSECKS expression increased chemotactic velocity and linear directionality, correlating with replacement of leading edge lamellipodia with fascin-enriched filopodia-like extensions, the formation of longitudinal F-actin stress fibers reaching to filopodial tips, relative enrichments at the leading edge of phosphatidylinositol (3,4,5)P₃ (PIP₃), Akt, PKC-zeta, Cdc42-GTP and active Src (Src^{poY416}), and a loss of Rac1.¹¹⁹ SSECKS is also thought to be a scaffold for protein kinase A, with resultant effects on angiogenesis¹²⁰ and a scaffold protein for protein kinase C, with downstream effects on the ERK pathway.^{121,122}

Apoptotic Pathway

Apoptosis is defined as a programmed cell death mediated by intrinsic or extrinsic pathways. The intrinsic pathway is

activated predominantly by intracellular stimuli, including DNA damage, oxidative stress and growth factor deprivation, and involves formation of apoptosome (comprised of procaspase-9, Apaf-1, and cytochrome *c*). The extrinsic pathway of apoptosis is initiated by the binding of death ligands (Fas ligands) to death receptors which leads to formation of death-inducing signaling complex (DISC). Following this, DISC can either directly induce cell death by activating effector caspases (caspase-3, -6, and -7) or cleave the Bcl-2 family member Bid, to activate the mitochondria-mediated intrinsic apoptotic pathway.

Caspase-8

Caspase-8 is a member of the cysteine-aspartic acid protease (caspase) family that is an effector of apoptosis. In neuroblastoma, greater metastases were observed in caspase-8-deficient tumors, whereas tumors expressing caspase-8 have less frequent metastases, highlighting caspase-8 role as a metastasis suppressor.¹²³ Interestingly, caspase-8-deficient cells showed no difference in tumor formation and growth *in vivo*. It was observed that caspase-8 expression was generally associated with increased apoptosis in highly invasive tumor cells,¹²³ suggesting a specific linkage of apoptotic and invasion pathways.

Growth Arrest Specific 1

Growth Arrest Specific 1 (GAS1) was isolated as a metastasis suppressor gene by a genome-wide RNAi screening in mouse melanoma cells.¹²⁴ Overexpression of GAS1 reduced spontaneous metastasis in melanoma cells by promoting apoptosis in the disseminated cancer cells at the secondary site.¹²⁴ Interestingly, expression of GAS1 could predict metastasis recurrence in stage II and III colorectal carcinoma.¹²⁵ Recent data on GAS1 mechanism of action highlight its independent roles in suppressing aerobic glycolysis and AMPK/mTOR/p70S6K signaling.¹²⁶

Transcriptional Complexes

Given the many intricate functions involved in the metastatic process, it could be expected that suppressors could exert multiple points of control, either through altered protein function or altered expression of proteins. Several examples of metastasis suppressors that act as transcriptional regulators have been reported.

Breast cancer metastasis suppressor 1

Breast cancer metastasis suppressor 1 (BRMS1) was metastasis suppressive in MDA-MB-231 and MDA-MB-435 breast cancer cells¹²⁷ without affecting the cancer cells growth *in vitro* or *in vivo*. Subsequently, BRMS1 metastasis suppression was also demonstrated in ovarian cancer, melanoma, non-small cell lung cancer, and bladder cancer.¹²⁸ BRMS1 affects several key events of metastasis, including inhibition of migration, invasion, and initiation of growth by single cells, and promotion of anoikis.^{129,130} BRMS1 overexpression also

restore homotypic and heterotypic gap junction's intercellular communication.¹³¹ A role for BRMS1 in transcriptional regulation was proposed by its identification in the mSin3 histone deacetylase complex, with a functional readout of transcriptional repression.¹³² In support of this hypothesis, the nuclear localization signal within BRMS1 was found to be necessary for metastasis suppression.¹³³ BRMS1 also poly-ubiquitinated p300, a histone acetyltransferase, and this activity was essential to its metastasis suppressor function.¹³⁴

MYC

MYC is a pleiotropic transcription factor that participates in the regulation of a wide variety of genes known to be involved in cell cycle, growth, apoptosis, cell adhesion, and genomic stability.^{135–137} MYC overexpression is widely known to transform cells and these cells can form tumors in experimental animals.^{138,139} Its overexpression has been reported in several cancer types.¹⁴⁰ A complete paradigm shift occurred when it was reported that overexpression of MYC stimulated proliferation of breast cancer cells (both *in vitro* and *in vivo*), but suppressed motility and invasiveness in culture.¹⁴¹ In xenografted tumors, MYC overexpression inhibited metastases to liver and lungs. Both the above phenotypes have been attributed to MYC-mediated repression of $\alpha\beta3$ integrin transcription.¹⁴¹ In an independent study, genetic or pharmacological inhibition of MYC synergized to increase TGF- β induced metastasis.¹⁴² A role for MYC control of protein translation is also implicated in its suppressive role.¹⁴³ MYC expression has been reported as a favorable prognostic factor.¹⁴⁴ MYC metastasis suppression is likely context dependent.^{145,146} This type of data provides a strong cautionary tale for the advancement of translational projects without metastasis data.¹⁴²

Anti-Metastatic miRNAs (Metastamirs)

miRNAs which are associated with the regulation of metastasis (pro or anti) are commonly referred to as Metastamirs/metastasis-associated miRNAs. Metastamirs are generally identified using *in vitro* screens for several of the metastatic cascade steps including EMT, adhesion, migration, invasion, apoptosis, and/or angiogenesis. Metastasis-suppressing Metastamirs include:

miR-146a/b

miR-146 was identified as a molecule responsible for the BRMS1 mediated metastasis suppression. Its expression is altered by BRMS1 and both are associated with decreased signaling through the NF κ B pathway. Transduction of miR-146a/b into MDA-MB-231 cells downregulated expression of EGFR and inhibited invasion, migration, and significantly suppressed experimental lung metastasis.¹⁴⁷

miR-335 and -206

In a search for microRNAs whose expression was gradually lost with increasing metastatic potential in human breast

cancer cells, these miRNAs were identified. Re-expression of these microRNAs in malignant cells suppressed lung and bone metastasis without affecting the size of primary tumor. miR-335-mediated suppression of metastasis targeted the progenitor cell transcription factor SOX4 and extracellular matrix component tenascin C.¹⁴⁸

The molecular identification of Metastamirs is in a nascent stage. With other miRNA projects, metastamirs have potential therapeutic implications through direct or indirect delivery.

Long Non-Coding RNA (lncRNA)

lncRNAs are long transcribed RNA molecules (greater than 200 nucleotides) and they do not have protein-coding capacity. They are transcribed by RNA pol II, are generally capped, polyadenylated, and frequently spliced.¹⁴⁹ lncRNAs are known to function by their interactions with proteins, RNAs, and DNAs.¹⁵⁰ They can also function as a transcriptional guide (recruitment of chromatin-modifying enzymes),¹⁵¹ a decoy (miR sponges)¹⁴⁹ and as a scaffold (stabilization of ribonucleoprotein complexes).¹⁵²

lncRNA LET

Its ectopic expression in hepatocellular carcinoma cells (SMMC-7721 and HCCLM3) and colon carcinoma (SW480) cells resulted in inhibition of lung colonization after tail vein injection.¹⁵³ lncRNA LET has been proposed to work by binding to NF90 (a double-stranded RNA binding protein), resulting in ubiquitination and degradation of NF90.¹⁵⁴

NF κ B-activating lncRNA

NF κ B-activating lncRNA (NKILA) is an inhibitor of metastasis in both *in vitro* and *in vivo* model systems and is reported to work by a protein–RNA interaction. It was identified as an lncRNA induced by NF κ B-signaling activating inflammatory cytokines (TNF α and IL-1 β) in breast cancer cells and acts as a negative regulator of NF κ B signaling.^{155,156} NKILA shows low expression in breast carcinomas (without distant or regional lymph node metastasis) and its expression was further reduced in metastasis and predicted a poor clinical outcome.¹⁵⁵ Ectopic expression of NKILA in MDA-MB-231 cells reduces invasion and in xenografts model inhibited metastasis to the lungs, liver, and lymph nodes and prolonged survival.¹⁵⁵

Metastasis Susceptibility Genes

These refer to group of genes that are associated with inherited susceptibility for the development of metastatic lesions.^{157,158} Metastasis susceptibility genes display a germline polymorphism which modifies tumor cell metastatic capability, indicating that heritable genetic variability can predetermine a tumor cell's propensity to metastasize. These genes were identified using a complex genetics screen that

exploits the differential heritable metastatic susceptibility observed among different strains of inbred mice.¹⁵⁹

Cadm1

Among several, Cadm1 is a validated member of the metastatic susceptibility genes group, overexpression of which specifically suppress metastasis without any significant difference in primary tumor growth. The molecular mechanism of Cadm1 involves sensitization of tumor cells to immune-surveillance mechanisms by CD8⁺ T cells.¹⁶⁰

Conclusions

We have attempted to survey the metastasis suppressor literature for evidence of their efficacy and underlying mechanisms. The very incomplete nature of the data available may stem from the difficulty of performing metastasis experiments, with no single *in vitro* assay. Based on the available literature, it can be inferred that majority of the metastasis suppressor effects are multifactorial and some may show their effect in context-dependent fashions. It is likely that, with continued gene expression and mutation profiling, additional candidate metastasis suppressors will be identified and validated.

Clinical application of metastasis suppressors is in nascent stage. It is currently proposed as a prognostic marker which can predict better or worse outcome based on recurrence.^{161,162} However, it is anticipated that, with the discovery of detailed mechanisms of action, new metastasis suppressor genes, translational approaches to enhance their activity, and better clinical trials design to target metastasis, the field can be of direct therapeutic importance.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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