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The role of tumor metastasis suppressors in cancers of breast and prostate

Eiji Furuta, Sucharita Bandyopadhyay, Megumi Iiizumi, Sonia Mohinta, Rui Zhan, and Kounosuke Watabe

Department of Medical Microbiology and Immunology, Southern Illinois University School of Medicine, 801 N Rutledge St, Springfield, IL 62702, USA

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1. ABSTRACT

Despite significant improvement in surgical techniques and chemotherapies, none of the current medical technologies “cure” metastatic disease, and the patients who have acquired metastatic cancer inevitably die from disseminated disease. Thus, there is a need for developing novel therapeutic approaches which can directly target metastatic tumor cells. However, advances in understanding the molecular mechanism of tumor metastases have lagged behind other developments in the cancer field. Tumor metastasis involves complex array of steps with each step requiring a coordination of the actions of many positive and negative factors. A number of tumor metastasis suppressors have been identified which suppress the formation of tumor metastasis without affecting the growth rate of the primary tumor. Such discoveries offer new approaches for curtailing tumor metastasis. This review summarizes our current understanding on these genes and their potential role in the progression of tumor metastases.

2. CLINICAL SIGNIFICANCE OF TUMOR METASTASES

Malignant tumors metastasize to adjacent or distant organs through the blood vascular circuit or lymphatic system. When cancer is detected at an early stage, before it has spread to other distant sites, it can be treated successfully by surgery or local irradiation and the patient will be cured. However, treatments are much less successful when the cancer is detected after it has already metastasized. Unfortunately, most patients present with a metastatic disease at the time of the first visit to the clinic, and in addition, many patients who do not present any evidence of metastasis at the time of their initial diagnosis, metastases will be detected at a later time. Therefore metastatic disease is a serious concern for survival of cancer patients. In spite of this clinical importance of metastasis, much remains to be learned about the biology of the metastatic process.

It is well known, based both on clinical observations and mechanistic studies, that metastasis formation is an inefficient process (1). Although large numbers of tumor cells are shed into the vascular drainage system from a primary tumor, it has been demonstrated experimentally that, after intravenous injection of highly metastatic tumor cells, approximately only 0.01% of these cells form tumor foci (2, 3). The inefficiency of tumor cells in completing the metastatic cascade results from the fact that successful formation of metastatic foci consists of several highly complex and interdependent steps. Each step is rate-limiting in that, failure to complete any of these events totally disrupts metastasis formation (1). The steps involved in metastasis formation are described below.

3. PROCESS OF TUMOR METASTASES

After the initial neoplastic transformation, the tumor cells undergo progressive proliferation that is accompanied by further genetic changes and development of a heterogeneous tumor cell population with varying degrees of metastatic potential. The oncogenic transformation is a result of the balance between the proto-oncogenes, which gain function by mutation, and the tumor suppressor genes, which contribute to tumorigenesis by loss of function (4, 5). The initial growth of the primary tumor is supported by the surrounding tissue microenvironment, which eventually becomes rate-limiting for further growth. As the tumor grows and the central tumor cells become hypoxic, the tumor initiates recruitment of its own blood supply. This process is referred to as the angiogenic switch and involves a balance between secretion of various angiogenic factors and removal or suppression of angiogenesis inhibitors (6, 7). The numerous positive and negative factors involved in angiogenesis are listed in Table 1. Notably, the process of neovascularization is almost invariably associated with a dramatic increase in the metastatic potential of tumors.

Continued genetic alteration in the tumor cell population results in selection of tumor cell clones with distinct growth advantage and acquisition of an invasive phenotype. Invasive tumor cells down-regulate cell-cell adhesion by modulating the expression of cadherins, alter their attachment to the extracellular matrix by changing integrin expression profiles and proteolytically alter the matrix by secretion of the matrix metalloproteases (1). Collectively, these changes result in enhanced cell motility and the ability of these invasive cells to separate from the primary tumor mass. These cells can detach from the primary tumor and create defects in the extra-cellular matrix that define tissue boundaries such as basement membranes, thus accomplishing stromal invasion. Furthermore, the poorly formed tumor vasculature that is generated in response to the angiogenic switch in the primary tumor mass, as well as thin walled lymphatic channels in the surrounding stroma, are readily penetrated by these invasive tumor cells and offer ready conduits to the systemic circulation (6). Endothelial cells responding to the angiogenic stimulus produced by the primary tumor also express an invasive phenotype and greatly enhance the metastatic process (7).

Once the tumor cells and the tumor cell clumps (emboli) have reached the vascular or lymphatic compartments, they must survive a variety of hemodynamic and immunologic challenges. Because cancer cells often express tumor specific antigens, they are attacked by non-specific (macrophage and NK cells) as well as specific (T cells) immune systems. However, some tumor cells evade the immune surveillance by a variety of mechanisms such as down-regulation of MHCI (8) and secretion of Fas ligand (9). After survival in the circulation, tumor cells must arrest in distant organs or lymph nodes. This arrest may occur by size trapping on the inflow side of microcirculation, or by adherence of tumor cells through specific interactions with capillary or lymphatic endothelial cells, or by binding to exposed basement membrane. In most cases, arrested tumor cells extravasate before proliferating. After exiting the vascular or lymphatic compartments, metastatic tumor cells may proliferate in response to paracrine growth factors or become dormant. After extravasation, tumor cells migrate to a local environment more favorable for their continued growth. Findings using *in vivo* video-microscopy demonstrate that the poor growth of tumor cells after extravasation from the circulation is a major factor contributing to the inefficiency of the metastatic process (10).

According to a century-old theory, a disseminated cancer cell acts like a seed, growing only if it finds suitable soil at a secondary site. Support for this idea comes from the observation that the target organ of metastasis is typically better than non-target organs in stimulating the growth of cancer cells *in vitro* (11). For example, researchers have noted that the bone marrow, in contrast to various other organs, strongly stimulates prostate cancer cell growth *in vitro* but has little or no effect on cancer cells that metastasize to non-bone organs (12). Similar correlations have been made for cancer cells *in vivo*. In a study of mammary cancer sublines with varying patterns of metastasis, the preferred organ of metastasis in each case was the organ allowing the most rapid growth of cancer cells (13). A traditional alternative to the “seed and soil” argument, known as the anatomical-mechanical hypothesis, challenges the importance of the soil in regulating cancer cell growth. It argues instead that metastasis develops in the organ of any capillary bed in which a disseminated cancer cell becomes mechanically lodged (11). Consistent with this hypothesis, it was noted in the 1940s that specific veins draining the prostate encountered their first capillary bed in the lumbar spine, which is a common site of prostate cancer metastasis (14). More recent findings also suggest that the cancer cell may have an important role in modifying the environment that it encounters. The environment reacts to this modification by inducing changes in the tumor cell and the cycle repeats (15). Hence, according to this model, the regulatory interaction between seed and soil is dynamic and reciprocal.

4. TUMOR METASTASES SUPPRESSOR GENES AND THEIR ROLES IN CANCER PROGRESSION

As described above, the process of tumor metastases involves multiple steps with high complexity and each step requires a coordination of the actions of

Table 1. Factors involved in the process of tumor metastases

Factor	Function	Expression in cancer	Location	Reference
Positive Factor				
Twist	Transcription, Cell adhesion	Breast, Prostate	7p21.2	109, 110
MMP2	Degrades extracellular matrix	Breast, Lung	16q13-q21	111, 112
MMP7	Degrades extracellular matrix	Colorectal, Gastric, Lung	11q21-q22	113-115
Catenin alpha 1	Cell signaling	Pancreatic	5q31	116
Catenin beta 1	Cell signaling	Breast, Prostate	3p21	117, 118
uPA	Serine protease	Breast, Prostate, Colorectal	10q24	119-121
Reptin	ATPase, DNA helicase activity	Prostate	19q13.3	118
VEGF	Angiogenesis	Breast, Prostate, Colorectal	6p12	121-123
PLGF	Angiogenesis	Breast	14q24-q31	124
FGF 1	Cell proliferation, Angiogenesis	Prostate	5q31	125
FGF 4	Cell proliferation, Angiogenesis	Prostate	11q13.3	125
TGF beta	Cell proliferation, differentiation	Breast, Prostate	19q13.1	126, 127
EGF	Cell proliferation, mitogenicity	Breast, Prostate	4q25	128, 129
PDGF	Embryological development	Breast, Prostate	22q13.1	130, 131
GCSF	Cell growth, Survival	Prostate	17q11.2-q12	132
IL-8	Angiogenesis	Breast, Prostate, Clorectal	4q13-q21	121, 133, 134
Angiogenin	Angiogenesis	Breast, Prostate	14q11.1-q11.2	135, 136
CD44	Cell adhesion, migration	Breast, Prostate	11p13	137
HGF	Cell growth, motility	Breast, Prostate, Lung	7q21.1	138-140
AMF	Glycolysis, Neurotropic factor	Breast, Prostate	19q13.1	141, 142
Snail homolog 2	Transcriptional repressor	Breast, Liver	8q11	143, 144
Negative Factor				
E-cadherin	Cell adhesion	Breast, Prostate, Lung	16q22.1	145-147
Fibronectin 1	Cell adhesion molecule	Breast	2q34	148
Vimentin	Cell adhesion molecule	Prostate	10p13	149
Thrombospondin 1	Angiogenesis	Breast	15q15	150
Angiostatin	Angiogenesis	Breast, Prostate	6q26	151, 152
Endostatin	Angiogenesis	Hepatoma	21q22.3	153
Vasostatin	Angiogenesis	Lung	14q32	154

many positive and negative factors. The fact that fusing a non-metastatic cell with highly metastatic cancer cell results in suppression of metastatic ability of the tumor cell raised a hypothesis that tumor metastasis is negatively regulated by tumor metastasis suppressor genes (16). They are defined as genes that suppress the formation of metastases, without affecting the growth rate of the primary tumor. Search for such genes using multiple approaches such as micro-cell mediated chromosome transfer (MMCT), microarray analyses and subtractive hybridization, has been quite effective, and to date, there are fourteen identified genes that clearly meet this criterion (Table 2). The following section summarizes the current information on each of these genes.

4.1. NM23

NM23 was the first gene isolated as a tumor metastasis suppressor. To identify a differentially expressed gene involved in tumor metastasis, Steeg *et al.* utilized a series of related murine melanoma cell lines of varying metastatic potential (17). By subtractive hybridization between the mRNAs from cell lines with low and high metastatic potential,the NM23 gene was isolated (17). They noted that NM23 mRNA levels did not correlate with cells' sensitivity to host immunological responses and therefore must be associated with intrinsic aggressiveness. In addition to the clinical observation of the down-regulation of NM23 gene expression in breast carcinoma (18), transfection of NM23 into highly metastatic breast, melanoma, colon, and oral squamous cell lines reduced *in vivo* metastatic potential of these cells (19-21). In addition, transfection of human NM23 into human breast carcinoma cells reduced *in vitro* motility to numerous attractants and inhibited colonization in soft agar (19). The metastasis suppressive activity of NM23 was previously correlated with its histidine protein kinase activity although physiological substrates for this unusual kinase activity have not been identified (22). Hartsough *et al.* reported that NM23 co-immunoprecipitated with the KSR (kinase suppressor of Ras) protein and phosphorylated ser-392 and ser-434 on KSR (23). It has been hypothesized that phosphorylation of KSR by NM23 alters its scaffold function, which could lead to reduced ERK activation in response to signaling. In agreement with this hypothesis, MDA-MB-435 breast cancer cells that over-express NM23 showed reduced ERK activation levels compared with vector alone control transfectants, while a histidine-kinase-deficient mutant of NM23 showed high levels of activated ERK, compared to those of the controlled transfectants (23). Therefore, altered levels of NM23 in metastatic versus non-metastatic tumor cells might impact ERK activation through a complex interaction with the KSR scaffold protein.

4.2. KAI1

The KAI1 gene was isolated originally by microcell mediated chromosome transfer technique (MMCT) as a prostate-specific tumor metastasis suppressor gene. It is located in the p11.2 region of human chromosome 11 (24, 25). When the KAI1 gene was transferred into a highly metastatic prostatic cancer cell line, KAI1-expressing cancer cells were suppressed in their metastatic ability, whereas their primary tumor growth was not affected (24, 25). Therefore, this gene behaves as a classical tumor metastasis suppressor. DNA sequencing analysis of the KAI1 gene revealed that it is identical to CD82, a surface glycoprotein of leukocytes, which encodes 267 amino acids (27). The protein has four hydrophobic and presumably transmembrane domains and one large extracellular N-glycosylated domain. Consistent with the view that KAI1

Table 2. Tumor metastases suppressor genes

Gene	Suppressed in cancer	Location	Function	*In vitro* Motility	*In vitro* Invasion	Tested in Animal	Immunohistochemistry (% negative in met patients)	Reference
Drg-1	Breast, Prostate Colon	22q12.2	Inhibit invasion	↓	↓	+	60% (P=0.04) (Breast), 74% (P=0.003) (Prostate)	102, 105, 106, 108
KAI1	Breast, Prostate	11p11.2	Integrin Interaction, EGFR desensitization	↓	↓	+	94.9% (P=0.025) (Breast), 100% (Prostate)	26, 29
BRMS1	Breast, Melanoma	11q13-q13.2	Gap junctional commiuncation	↓	↓	+		49, 50
KiSS-1	Breast, Melanoma	1q32-q41	G-protein-coupled receptor ligand	↓	↓	+	56% (P=0.482) (Melanoma)	43, 155
NM23	Breast, Prostate Melanoma, Colon	17q21.3	Histidine Kinase	↓	↓	+	66.7% (P=0.013) (Breast), 73% (P=0.289) (Prostate)	17, 156-158
RhoGDI2	Bladder	12p12.3	Regulates Rho & Rac function	↓	↓	+		89
CRSP3	Melanoma	6q22.33-q24.1	Transcriptional coactivator	↓	↓	+		64
MKK4	Prostate, Ovary	17p11.2	MAPKK, JNK kinases	↓	↓	+	67.7% (P<0.0001) (Ovary)	39, 42
VDUP1	Melanoma	1q21.1	Thioredoxin inhibitor					64
E-Cadherin	Breast, Prostate Gastric, Colorectal, Thyroid, Ovary	16q22.1	Inhibit shedding from primary tumor		↑↓	+	47.7% (P=0.147) (Breast), 27.3% (P=0.004) (Prostate)	55, 159, 160
RKIP	Breast, Prostate, Melanoma	12q24.23	Inhibits Raf-mediated MEK phosphorylation	↓	↓	+	39.2% (p=0.367) (Breast)	66, 161
SSeCKS	Prostate	6q24-25.2	Scaffolding protein for PKC & PKA	↓		+		72
Claudin 7	Breast, Cervical, Gastric	17p13	Tight junction protein					76
RRM1	Lung	11p15.5	Ribonucleotide reductase	↓	↓	+		80, 82

is a metastasis suppressor gene, the immunohistochemical analysis of human tumor samples revealed that the expression of the gene in most cases was downregulated during the tumor progression of not only prostate, but also lung (28), breast (29), bladder (30), and pancreatic cancers (31). The down-regulation of the KAI1 gene expression is also correlated with poor survival in patients with those cancers. Furthermore, in a study of prostate tumors including 120 cases, PCR-single-strand conformational polymorphism and microsatellite analyses revealed that the KAI1 expression was down-regulated consistently during the progression of human prostatic cancer and that this down-regulation did not commonly involve either mutation or allelic loss of the KAI1 gene (26). Therefore, the expression of this gene appears to be down-regulated in advanced tumor cells at or post-transcriptional level, presumably by the loss of an activator or gain of a suppressor.

In order to understand the basic regulatory mechanism of the KAI1 gene expression, the 5' upstream region of the KAI1 gene was cloned by screening a human placental genomic library in our laboratory (32). The KAI1 promoter revealed a p53 consensus binding site and in addition, reverse transcription-PCR analysis revealed that the expression of endogenous KAI1 mRNA was augmented significantly by p53. The results of the promoter analysis using a reporter plasmid containing the 5' upstream sequence indicated that the KAI1 gene was indeed positively controlled by p53 at the transcriptional level in prostatic tumor cells. By subsequent analysis of the promoter sequence of the KAI1 gene by site specific mutagenesis and gel-shift mobility assay, we found that the region of 272 bp, which was approximately 860 bp upstream of the transcriptional initiation site, was responsible for this p53 activation (32). Results from these experiments clearly indicate that p53 activates the KAI1 gene at the transcriptional level through its binding to the specific site of the 5' upstream region.

In the search for a specific agent which re-activates the expression of the KAI1 gene, it was found in our laboratory that etoposide, a topoisomerase II inhibitor, is able to activate the expression of the KAI1 gene in a dose-dependent manner in human prostate cancer cell lines as well as in human lung carcinoma cells (33). Our results suggest that the augmentation of the KAI1 gene expression by etoposide is independently controlled by both p53 and c-Jun at the transcriptional level in the human prostate tumor cell lines. Furthermore, treatment of these cell lines with etoposide resulted in a significant reduction of cellular invasion (33). Because etoposide has been shown to be effective on advanced prostate cancer when used in combination with other regimens, our results provide a further rationale to use this drug as an anti-metastatic agent.

How the KAI1 gene suppresses the metastasis process remains the most intriguing question. Recently, Odintsova *et al.* found that KAI1 physically associates with the EGF receptor and rapidly desensitizes the EGF-induced signal that could lead to suppression of cell migration (34). However, it is yet unclear whether this mechanism indeed accounts for the metastasis suppression *in vivo*. The crucial clue to understand the biochemical function of the KAI1 gene came from the results of the recent studies on T-cell activation. KAI1/CD82 is barely detectable on resting peripheral T and B lymphocytes, while its expression is highly up-regulated upon activation of these cells (35). This up-regulation is associated with some morphologic change and expression of activation markers such as CD82 and MHC II antigens. Lebel-binay *et al.* described that the co-engagement of KAI1/CD82 and TCR by anti-CD82 mAb and anti-CD3 mAb, respectively, was able to activate T cell and that, when a T-cell is stimulated *in vitro* by anti-KAI1/CD82 mAb, KAI1/CD82 appears to transmit a signal which results in tyrosine phosphorylation, a rapid increase in intracellular Ca^{2+} level and IL-2 production (36). Interestingly, this activation was associated with a change in cellular morphology and inhibition of cell proliferation (37). Therefore, it is tempting to speculate that tumor cells of epithelial origin may also employ a similar signal pathway upon activation of KAI1/CD82, which results in growth arrest of tumor cells. In fact, it was shown that NGF was capable of up-regulating the expression of KAI1 in prostate cancer cell lines, and this activation was associated with remarkable down-regulation of cell proliferation *in vitro* and *in vivo* (38). Although it remains to be tested whether the KAI1 up-regulation is coupled to the inhibition of cell proliferation, this raises an attractive possibility that activation of KAI1 may lead to growth suppression in tumor cells of epithelial origin similar to that in cells of haematopoetic origin under certain conditions. Thus the existing information points to a very diverse mode of activation of KAI1/CD82 as revealed in the *in vitro* experiments.

4.3. MKK4

The MKK4 gene was originally identified as a metastasis suppressor for prostate cancer by combination of MMCT and differential expression approaches (39). Following identification of metastasis suppressor activity of a 70cM region on human chromosome 17 in an *in vivo* animal model (40), Yoshida *et al.* examined the genes located within this region and having a biological function suggesting a potential role in metastasis suppression (39). Putative candidate genes that were not specifically retained or expressed by microcell mediated chromosome 17-tranferred prostate cancer cells and normal prostate tissue were eliminated from further consideration. MKK4/SEK1 was identified as a candidate gene based on its physical location, 17p11.2, within the 70-cM metastasis suppressor region, and the fact that its normal cellular function in the stress-activated signaling pathway suggests that alteration of this gene may have pleiotrophic effects on the cell (39). The same group of investigators also observed that expression of the MKK4 gene in a metastatic prostate cancer cell line significantly reduced the number of macroscopic lung metastases in SCID mice as compared with the lungs from control animals, without affecting the primary tumor growth (39). Detailed histological examination of sections from the lungs of tumor-bearing animals indicated that lungs from control mice had large metastatic foci while the lungs from mice bearing MKK4-positive tumors contained significantly small foci. In addition, cuffs of cells approximately two to three layers thick were observed around blood vessels in several of the sections from the MKK4-positive samples, suggesting that the tumor cells may co-opt existing host vasculature for growth (39).

In order to understand the clinical significance of the MKK4 gene in cancer progression, Kim *et al.* performed immunohistochemical studies on clinical samples of prostate cancer (41). The study revealed high levels of MKK4 expression in the epithelial but not the stromal compartment of normal prostatic tissues with a significant down-regulation of expression in the neoplastic tissues, and a statistically significant inverse relationship between Gleason pattern and MKK4 was observed (41). These results demonstrate that the MKK4 gene is consistently down-regulated during prostate cancer progression and supports the notion that disregulation of the MKK4 signaling cascade plays a crucial role in progression of metastatic disease. Similar results have been reported for ovarian cancer as well (42). To test the possibility that down-regulation of MKK4 protein is the result of allelic loss, Kim *et al.* examined the metastatic prostate cancer lesions for loss of heterozygosity (LOH) within the MKK4 locus and found that the downregulation of MKK4 expression in cancer patients does not frequently involve allelic loss or mutation of this gene (41). Although MKK4 is a central molecule in the cell's stress response pathway, how this gene inhibits the metastasis process is yet to be understood.

4.4. KiSS-1

The KiSS-1 gene was originally identified as a metastatic melanoma suppressor gene by combining the aspects of the strategies of both MMCT and differential display. After the introduction of human chromosome 6 into human metastatic melanoma cell lines C8161 or MelJuSo by MMCT resulted in a significant suppression of metastasis without affecting tumorigenicity or local invasiveness, a subtractive hybridization between the highly metastatic parental C8161 and the chromosome 6-C8161 hybrid cells led to the identification of the KiSS-1 transcript (43). The functional role of KiSS-1 in metastasis suppression was evident when the full-length KiSS-1 transfectants suppressed the lung colonization of tumor cells in spontaneous metastasis assay without affecting the growth of the tumor cells *in vivo* (43). Based on the observation that chromosome 1q is frequently deleted in late-stage human breast carcinomas, Lee *et al.* tested whether the KiSS-1 gene that maps to chromosome 1q32-

q41 could suppress metastasis of the human breast carcinoma cell line MDA-MB-435 (44). They found that the expression of KiSS-1 almost completely abrogated the metastatic potential as compared to control cells but did not suppress tumorigenicity. Therefore, KiSS-1 acts as a metastasis suppressor for breast carcinoma as well. The same investigators also noted that metastasis suppression by KiSS-1correlated with a decreased three-dimensional growth of cells in soft agar but invasion and motility were unaffected. Based on the predicted structure of the KiSS-1 protein, these results imply a mechanism whereby KiSS-1 regulates events downstream of cell-matrix adhesion, perhaps involving cytoskeletal reorganization.

Yan *et al.* have recently found that colon carcinoma cell lines HT-1080 stably transfected with a KiSS-1 expression construct, demonstrated substantially lower MMP-9 enzyme activity and *in vitro* invasiveness (45). The lower MMP-9 enzyme activity reflected reduced steady-state mRNA level that in turn was due to attenuated transcription. Moreover they noted that while activation of ERKs and JNKs by phorbol 12-myristate 13-acetate and tumor necrosis factor alpha, respectively, were able to increase the MMP-9 expression, this MMP-9 activation was not antagonized by KiSS-1 expression, suggesting that MAPK pathways modulating MMP-9 synthesis are not the target of KiSS-1 (45). They further observed that although MMP-9 expression is regulated by AP-1, Sp1 and Ets transcription factors, KiSS-1 did not alter the binding of these factors to the MMP-9 promoter. However, NF-κβ binding to the MMP-9 promoter required for expression of this collagenase was reduced by KiSS-1 expression. Diminished NF-κβ binding reflected less p50/p65 in the nucleus secondary to increased I-κβ levels in the cytosols of the KiSS-1 transfectants (45). Their results suggest that KiSS-1 diminishes MMP-9 expression by effecting reduced NF-κB binding to the promoter. Another important clue for KiSS-1 function came from the study of Ohtaki *et al.* (46), who isolated a 54 amino acid peptide from human placenta that turned out to be encoded by Kiss-1 C-terminus and served as the endogenous ligand for an orphan G-protein-coupled receptor (hOT7T175). Named as 'Metastin', this peptide inhibits chemotaxis and invasion of hOT7T175-transfected CHO cells *in vitro* and attenuates pulmonary metastasis of hOT7T175-transfected B16-BL6 melanomas *in vivo*. These results suggest possible mechanisms of action for KiSS-1 and a potential new therapeutic approach. Interestingly, since then, similar results have been reported by two other groups independently (47, 48).

4.5. BRMS1

Several regions spanning the q-arm of chromosome 11 have been found to be associated with a majority of breast cancer cases, the most common being amplifications and deletions involving regions near band 11q13 (49). In particular, reports of high-frequency deletions involving 11q13-q14 in late-stage, metastatic breast carcinomas were suggestive of the existence of a metastasis suppressor gene in this region (49). This was further corroborated by the finding that introduction of a normal human chromosome 11 into the metastatic MDA-MB-435 human breast carcinoma cells by microcell-mediated transfer significantly suppressed metastasis without affecting tumorigenicity. Then, DD-RT-PCR for highly metastatic (MDA-MB-435) parental cells versus the metastasis-suppressed clones led to the identification of three novel cDNA fragments, one of which was identified as BRMS1 (50). Over-expression of BRMS1 in metastatic breast carcinoma cells suppressed metastasis in both spontaneous and experimental breast cancer metastasis models (50). In addition, the same gene was also found to act as a metastasis suppressor for melanoma (51). Stable transfection of BRMS1 in the human melanoma cell lines MelJuSo and C8161.9 did not alter the tumorigenicity of either cell line, but significantly suppressed metastasis compared to vector-only transfectants (51). However, the expression of this gene has not yet been examined in clinical setting.

Toward analyzing mechanisms underlying suppression of metastasis by BRMS1, Samant *et al.* observed that expression of BRMS1 in tumor cells did not make significant difference in adhesion to extracellular matrix components (laminin, fibronectin, type IV collagen, type I collagen) or invasion and only modestly inhibited the motility of the cells and, in some cases, inhibited the ability of the cells to grow in three-dimension in soft agar (52). The results of their study also ruled out the possibility of BRMS1 upregulating expression of other metastasis suppressors, such as NM23, KAI1, KiSS1 or E-cadherin. Some clue regarding function of BRMS1 came from a study by Saunders *et al.*, who reported that transfection and re-expression of BRMS1 restored the ability of human breast carcinoma cells (MDA-435) to form functional homotypic and heterotypic gap junctions (53). Cx43 and Cx26 (connexins) are the predominant gap junction protein in normal breast epithelial tissue but are often reported to be lost in neoplastic breast tissue. Metastatic MDA-MB-435 cells express Cx32 but not Cx43 or Cx26, and restoring BRMS1 expression in this cell line resulted in re-establishment of gap junction but only partly restored Cx43 expression. Based on these observations Saunders *et al.* suggested that re-expression of the BRMS1 gene restores the Cx expression profile from that of a metastatic cell to that more similar to a normal breast epithelial cell and that the composition of gap junctions contributes to metastatic propensity (53).

4.6. E-cadherin

The transmembrane protein E-cadherin (also known as CDH 1) was originally isolated as human uvomorulin by screeing a cDNA library of the human liver (54). The E-cadherin is a calcium-dependent adhesion molecule and constitutes a main component of the adherence junction in epithelia cells. Calcium ions bind to the extracellular domain of E-cadherin at the adhesion site of cell-cell junction, while the intracellular domain of this molecule interacts with beta-catenin to mediate actin binding. E-cadherin also sequesters the function of beta-catenin by blocking nuclear translocation which results in inhibition of transcription of c-myc and cyclin D1 (55). The expression of E-cadherin is generally reduced in a variety of human cancers at advanced stages. It is believed that tumor cells with a low level of E-cadherin can be readily detached from adjacent cells, and these cells invade and metastasize to other distant organs. Several groups have indeed reported that decreased expression of E-cadherin was associated with a poor prognosis of cancer patients (56). Most importantly, over-expression or maintenance of E-cadherin in invasive cancer cells has been shown to decrease motility and invasiveness (55). Therefore, E-cadherin is considered to function as a metastasis suppressor. Interestingly, E-cadherin has recently been found to be regulated by Snail and Slug (57) that are zinc-finger transcription factors and involved in the process of cell differentiation and apoptosis (58). In breast carcinomas, Snail and Slug have been recently shown to be involved in tumor progression and invasiveness (57), and it is postulated that these proteins repress the expression of E-cadherin (57).

4.7. VDUP1 (TXNIP) and CRSP3

The VDUP1 (Vitamine D3 upregulated protein 1) gene was first identified by the differential display technique as a gene induced by 1,25dihydroxyvitamin D-3 (59). VDUP1 is able to interact with a reduced form of TRN (60), which results in inactivation of TRN. TRN is an inhibitor for apoptosis signal-regulating kinase 1 (ASK-1) which is known to be a central component of stress-induced apoptosis (61). Therefore, VDUP1 is also considered to participate in this signal pathway through the binding to TRN (62). In fact, the expression of VDUP1 has been shown to arrest cell growth of NIH3T3 cells (63). Consistent with these *in vitro* results, immunohisotchemical analyses for tumor specimens revealed that the expression of VDUP and TRN were inversely correlated in many tumors. Over-expression of VDUP1 in a metastatic cell line followed by injection into mice significantly reduced the incidence of lung metastases, suggesting that VDUP1 functions as a metastasis suppressor, The regulatory mechanism of the VDUP1 gene has not been well understood, however, Goldberg and colleagues recently found that VDUP1 is controlled by a transcription factor, CRSP3, and suggested that CRSP3 may also act as an metastasis suppressor and as an up-stream regulator of VDUP and KiSS-1 in human melanoma (64). CRSP3 is known as a co-factor in Sp1 (Specificity protein 1) mediated transcription, and transfection of an expression plasmid of CRSP3 into melanoma cells significantly increased the expression of KiSS1 and VDUP1 genes. Consistent with the notion that CRSP3 is a metastases suppressor gene, over-expression of the CRSP3 gene in metastatic melanoma cells and transplantation of these cells into mice significantly decreased the rate of lung metastasis. Furthermore, the expression of VDUP1 and CRSP3 genes has been shown to be inversely correlated with the progression of melanoma by using quantitative real-time RT-PCR. Therefore, both VDUP1 and CRSP3 apparently act as metastases suppressors via the KISS1 pathway. However, mechanism of metastases suppression by these genes is not yet clear.

4.8. RKIP

Raf kinase inhibitor protein (RKIP) is a member of the phosphatidylethanolamine binding protein (PEBP) family. RKIP encodes a protein which inhibits the Raf/mitogen-activated protein kinase /extracellular signal-regulated kinase (ERK) pathway. This signaling plays an important role in determining cell fate and choosing between diverse responses such as proliferation, differentiation and survival. Interestingly, RKIP was recently identified as a gene significantly down-regulated in a metastatic cell line (C4-2B) of prostate cancer by microarray analyses (65). This result was further corroborated by immunohistochemical examination of clinical tissue samples from cancer patients. It was found that RKIP was usually expressed in benign tissues while it was significantly down-regulated in tumors, especially in metastatic cells. These results suggest that RKIP is associated with suppression of metastasis. In consistence with these data, over-expression of RKIP in a metastatic cell line derived from prostate cancer has been shown to have no effect on cell proliferation or colony-formation ability in soft agar but significantly lower the invasive potential of these cells. Furthermore, overexpression of RKIP drastically decreased the lung metastases of these cells when transplanted into animals without affecting primary tumor growth (66).

Since RKIP is an inhibitor of Raf which phosphorylates MEK and ERK, Fu *et al.* examined the status of phosphorylation of these target proteins in various prostate cancer cell lines and found that both MEK and ERK had higher basal levels of the phosphorylated forms in metastatic cells than in non-metastatic cell line, without significant changes in the total protein level (66). Conversely, the degree of phosphorylation of these target proteins was lower in metastatic cell with RKIP over-expression than in mock transfected cells. In this context, it should be noted that treatment of a metastatic cell line with a MEK kinase inhibitor significantly reduced the invasiveness of the cells, suggesting that RKIP suppresses tumor invasion through MEK activity (66). Interestingly, RKIP has also been shown to promote apoptosis of cancer cell, and low level of RKIP expression significantly increases resistance to chemotherapeutic-induced apoptosis. Thus RKIP also appears to contribute to response of cancer cells in chemotherapy (67).

4.9. SSeCKS

SSeCKS (*Src*-Suppressed C Kinase Substrate) was originally isolated by using PCR-based subtractive hybridization (68, 69). Over-expression of the SSeCKS gene via a retroviral vector caused a significant reduction in cell proliferation compared to a normal control cell or *src*-transfected cell, suggesting that SSeCKS encodes a regulator of mitogenesis. SSeCKS was also known as an orthologue of human Gravin/AKAP12 (A kinase anchor protein 12) which was previously identified as a cytoplasmic antigen recognized in sera from patients with myasthenia gravis (70) and later found to be the cytoplasmic scaffolding protein for protein kinase A and C (71, 72). Recently, Xia *et al.* showed that both RNA and protein levels of SSeCKS/Gravin were significantly decreased in metastatic prostate cancer cell lines of human and rat origin compared to non-metastatic cell lines (72). They also found that the expression of SSeCKS/Gravin inhibited anchorage-independent growth without affecting the cell proliferation. Furthermore, over-expression of

SSeCKS/Gravin in metastatic cell line followed by injecting it into mice significantly decreased the incidence of lung metastasis. Therefore, SSeCKS/Gravin appears to function as a metastasis suppressor.

4.10. Claudin

Claudins, a family of integral membrane proteins, are the basic molecules involved in tight junction structure and function (73). Tight junctions are responsible for controlling the paracellular permeability, cell adhesion and cell polarity. These functions of tight junctions that are often lost in cancer may play a crucial role in tumor growth and metastasis (74). Claudins as prime constituents for tight junctions have been found to be abnormally regulated in human breast and prostate cancers. Claudin-3 and claudin-4 are typically over-expressed in adenocarcinomas including prostate and breast cancers. On the other hand, recent study with pancreatic cancer suggests that claudin-4 functions as an inhibitor of the invasiveness of cells (75). Interestingly, claudin-7 has been found to be significantly down-regulated in invasive ductal carcinomas (IDC) of the breast and there is an inverse correlation between the expression of claudin-7 and cellular discohesion in breast carcinomas (76). These results suggest that claudin-4 and 7 are putatuve metastasis-suppressors, although the role of claudin-4 in the metastasis process remains to be clarified further.

4.11. RRM1

RRM1 (ribonucleotide reductase M1 polypeptide) encodes the regulatory subunit of ribonucleotide reductase which is known to catalyze the rate limiting step of deoxyribonucleotide formation (77-79). RRM1 is located on chromosome 11p15.5 which is often lost in lung cancer at advanced stages and is also significantly associated with metastatic spread in lung cancer patients (80, 81). A recent study by Bepler and colleagues showed that over-expression of RRM1 induced expression of the known tumor suppressor gene, PTEN, in human and mouse cell lines, and also in animal model (82). These authors found that a lung derived stable cell line over-expressing RRM1 significantly reduced migration and invasive abilities compared with a control cell line. The overexpression of RRM1 also strongly induced the expression of PTEN in these cell lines. Importantly, the expression of RRM1 suppressed spontaneous metastasis to the lung and prolonged survival in animals. Therefore, RRM1 appears to function as a metastasis suppressor through induction of PTEN in lung cancer. In fact, immunohistochemical analyses of clinical samples revealed that the expression of RRM1 was significantly correlated with PTEN and RRM2 (ribonucleotide reductase M2 polypeptide) (83). Furthermore, high expression of RRM1 was found to be predictive of long survival independent of tumor stage, performance status, and weight loss (83, 84).

4.12. RhoGD12

The Rho proteins belong to a guanine nucleotide family and they exist in two different forms as being active when bound to GTP and inactive when bound to GDP. RhoGDIs (GDI: GDP-dissociation inhibitor) are the class of proteins that inhibit the dissociation of GDP and stabilizes the inactive form of Rho proteins. RhoGDI2 is a 200 amino acid protein with a molecular weight of 229 kDa and it was first discovered by Leffers *et al.* (85). It was found to be expressed in human and murine hematopoietic tissues, predominantly in B and T lymphocytes (86) as well as in non-hematopoietic neoplastic cells (87). RhoGDI2 is phosphorylated in response to stimulation of T lymphocytes and myelomonocytes cells, and it is involved in inducing hematopoiesis (88). On the other hand, recent study of Gildea *et al.* (89) has shown that inducible expression of exogenous RhoGDI2 in metastatic cells blocked lung metastasis and significantly suppressed invasiveness and motility of cultured cells but did not affect the *in vitro* growth rate, colony formation or *in vivo* tumorigenicity. The intricacy of mechanism by which RhoGDI2 restricts metastasis is yet to be elucidated, but it is speculated that RhoGDI2 suppresses the metastatic process by impeding the tumor cells from invading and colonizing the lung upon reaching the pulmonary vasculature. RhoGDI2 has also been identified as a potent metastatic suppressor in bladder cancer. Therefore, RhoGDI2 is considered as a general metastases suppressor.

4.13. Drg-1

The Drg-1 gene was originally found to be induced *in vitro* by cellular differentiation and hence named as Differentiation-Related-Gene-1 (90). Since then, three more genes, namely, Drg-2, 3 and 4 have been identified that encode proteins highly related to Drg-1 (91, 92). These genes constitute the NDRG gene family although the members vary in the pattern of tissue-specific expression and possibly in function. Drg-1 is identical to the human RTP, cap43 and rit42, and homologous to the mouse genes TDD5 and Ndr1 and rat Bdm1 (93-98). The protein encoded by the Drg-1 gene has a molecular weight of 43 kDa and possesses three unique 10-amino acid tandem repeats at the C terminal end. Analysis of the amino acid sequence predicted that there were seven or more phosphorylation sites, and Drg-1 indeed has been shown to be phosphorylated by Protein Kinase A *in vitro* (99). Drg-1 mRNA is detected in most of the organs, and the level of expression is particularly high in prostate, ovary, intestine and kidney. It was shown that the expression of this gene was repressed by c-myc and N-myc/Max complex *in vitro* (97). On the other hand, p53 was found to be able to induce expression and nuclear translocation of Drg-1 in response to DNA damaging agents (95). The expression of the gene was also augmented by hypoxia and PTEN, and the combination of Drg-1 and PTEN has indeed been shown to be an indicative marker for outcome in patients with both breast and prostate cancers (100-102). In addition, the Drg-1 gene has been shown to be upregulated by hormones such as androgen (96) and by various chemical agents including homocysteine, mercaptoethanol, tunicamycin (98), lysophosphatidylcholine (103), nickel compounds (94) and synthetic retinoids (104). Therefore, the Drg-1 gene is controlled by multiple factors and responsive to various stimuli.

Table 3. Relationship between Drg-1 and other clinical parameters in prostate cancer

Drg-1 expression				
	All	Positive	Reduced	P value
Gleason grade				
≤ 7	38	26	12	
> 7	24	8	16	0.015 [1]
P53				
Wild type	59	32	27	
Mutant	3	2	1	0.8
Differentiation				
Well	16	14	2	
Moderate	19	14	5	
Poor	27	6	21	<0.001 [1]
Nuclear grade				
I	32	22	10	
II / III	30	12	18	0.044 [1]
Metastasis status				
Organ confined	40	28	12	
Lymph node	20	5	15	0.003 [1]
Bone	19	5	14	0.006 [1]

[1] Statistically significant. Ref 62

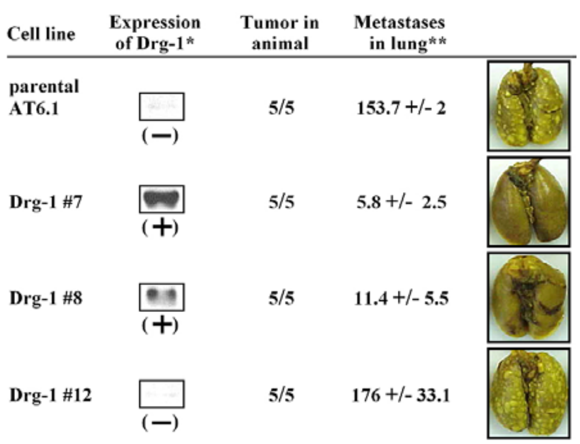

Figure 1. Drg-1 suppresses spontaneous lung metastasis without affecting growth of primary tumor. The parental cell line (AT6.1) and Drg-1-transfected clones (#7, #8, and # 12) were tested for Drg-1 protein expression by Western blot. Each of these cell lines was injected subcutaneously into SCID mice. After 4 weeks, the mice were sacrificed and the lungs were removed. The tumor nodules on the lungs were counted macroscopically. The lungs from mice from each group are shown as examples.

Since the Drg-1 gene is strongly correlated with differentiation and tumor progression is invariably associated with loss of differentiation, we analyzed the Drg-1 expression status in clinical samples of human prostate and breast cancer (105, 106). In both cases, Drg-1 was found to be highly expressed in the epithelial cells of normal glands and ducts where the protein was localized mostly in the cytoplasm. The Drg-1 protein was detected consistently in all cases of normal prostate tissue as well as PIN (Prostatic Intraepithelial Neoplasia) and BPH (Benign Prostatic Hyperplasia), and normal mammary gland cells, while the Drg-1 expression was significantly reduced in the tumor cells of cancer patients (105, 106). In the case of prostate cancer, the reduction in Drg-1 expression correlated significantly with the Gleason grade. A study by Caruso *et al.* also found similar trend of downregulation of Drg-1 expression in prostate cancer, and interestingly, they also observed a significant correlation between Drg-1 expression pattern and ethnic origin of the patients (107). Most interestingly, in both prostate and breast cancers, we observed a significant level of differential expression of Drg-1 between the patients with organ-confined disease and those with metastasis to lymph node or bone (Table 3,106). In case of prostate cancer, the negative correlation of Drg-1 with metastatic spread to lymph node and bone is highly significant, and in fact, is much stronger than the positive correlation with Gleason scores. In breast cancer, a similar and significant negative correlation of Drg-1 with metastases has been observed (106). These results strongly suggest the negative involvement of Drg-1 in the process of invasion and metastasis in both prostate and breast cancer.

The significant inverse correlation of Drg-1 expression with the extent of metastasis at the clinical level raised the next important question as to whether the down-regulation of Drg-1 is cause or result of metastases. To address this issue, we over-expressed the Drg-1 gene in a highly metastatic prostate cell line and implanted it into SCID mice. The result of this experiment indicated that all the clones formed primary tumors in the animals with similar growth rates (data not shown), suggesting that Drg-1 does not have an effect on tumorigenesis and tumor growth. On the other hand, the clones that were positive for Drg-1 expression exhibited a significantly lower incidence of lung metastases compared with the vector-transfected cell line (Figure 1). Similar metastasis suppressor effect of Drg-1 was also observed in colon carcinoma cells by Guan *et al.* (108). Furthermore we observed that Drg-1 significantly suppressed the invasive potential of prostate and breast cancer cells as tested by *in vitro* invasion chamber assay (105, 106). Therefore, evidence from both clinical data and the results of *in vitro* as well as animal experiments overwhelmingly support the notion that Drg-1 is a metastasis suppressor gene and that the down-regulation of the gene results in acceleration of tumor metastasis. How Drg-1 suppresses the tumor metastases is an intriguing question which is under active investigation.

5. CONCLUSION AND FUTURE DIRECTIONS

The development of metastases is a major obstacle to the successful treatment of a patient with any cancer. Much of the lethality of malignant neoplasms is directly attributable to their ability to develop secondary growths in organs at a distance from the primary tumor mass, while few patients die from their primary neoplasm. Although the clinical importance of tumor metastasis is well recognized, advances in understanding the molecular mechanism involved in metastasis formation have lagged behind other developments in the cancer field. This is because of the fact that metastasis involves multiple steps with high complexity. A possible breakthrough in our understanding of cancer progression has emerged with the hypothesis that tumor metastasis is negatively controlled by tumor metastasis suppressor genes. Thus far fourteen genes have been identified that are defined as tumor metastases suppressors. Almost all of them are also significantly down-regulated in advanced stages in a variety of cancers. However the mechanism of metastases suppression for most of the genes is yet to be clarified. A cross-talk between these proteins remains an intriguing question. The mechanism of down-regulation of these genes in tumor cells also needs to be addressed. Recent studies in this field have begun to shed light on these questions and understanding the molecular mechanism of tumor metastases suppression would eventually lead to the development of therapeutic approaches to intervene in the process of metastatic disease.

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Send correspondence to: Dr Kounosuke Watabe, Department of Medical Microbiology and Immunology, Southern Illinois University School of Medicine, 801 N Rutledge St, Springfield, IL 62702, USA, Tel: 217-545-3969, Fax: 217-545-3227, E-mail: kwatabe@siumed.edu

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