Review

Molecular biology of breast cancer metastasis
Genetic regulation of human breast carcinoma metastasis

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Abstract

The present is an overview of recent data that describes the genetic underpinnings of the suppression of cancer metastasis. Despite the explosion of new information about the genetics of cancer, only six human genes have thus far been shown to suppress metastasis functionally. Not all have been shown to be functional in breast carcinoma. Several additional genes inhibit various steps of the metastatic cascade, but do not necessarily block metastasis when tested using *in vivo* assays. The implications of this are discussed. Two recently discovered metastasis suppressor genes block proliferation of tumor cells at a secondary site, offering a new target for therapeutic intervention.

Keywords: *BRMS1*, E-cadherin, *KAI1*, *KiSS1*, *MKK4*, *Nm23*, metastasis-suppressor genes

Introduction

Colonization of distant tissues by tumor cells represents the most dangerous attribute of cancer. When breast carcinomas remain confined to breast tissue, cure rates exceed 90%. As cells spread, however, long-term survival decreases depending upon the extent of and the sites of colonization. Metastases in visceral organs and brain are the most life-threatening, with 5-year survival rates usually less than 20% [1]. Thus, in order to increase survival, prevention of metastasis and more effective treatment of already established metastases are necessary. Both will be possible only after we have a more thorough understanding of the biologic, biochemical, and molecular basis of cancer spread. Although the focus of the present review is on the genes that regulate metastasis, the context under which those genes operate is briefly addressed.

Metastasis is defined as the progressive growth of cells at a site that is discontinuous from the primary tumor. The route of spread is irrelevant in this definition. Cells can disperse via blood vasculature, lymphatics, or within body cavities. Metastatic cells are a specialized subset of tumor cells within a primary tumor mass that have acquired the ability to complete the multistep metastatic cascade (for review [2,3•,4–7]). In brief, these cells migrate, disseminate, extravasate, and eventually proliferate at a discontinuous secondary site(s). If a cell fails to complete any step in the metastatic cascade, then it is not metastatic.

MMP = matrix metalloproteinase; NDP = nucleoside diphosphate; TGF = transforming growth factor.

Failure to metastasize can be due to inherent deficiencies within tumor cells themselves (ie genetics) or to defective responses to the host environment (ie epigenetics). This concept is not new. Indeed, Paget [8•] advanced the notion over a century ago when he documented the nonrandom distribution of breast carcinoma metastases. Paget's explanation was that the tumor cell or 'seed' would grow only when cultivated in an appropriate organ or 'soil'. His agrarian analogy seems quaint in this era of molecular biology and genomics, but the fundamental principle has withstood the test of time; formation of metastasis depends upon interaction between tumor cells and host cells. Recently, some of the molecules that are responsible for organ-specific colonization have begun to be elucidated [9–13].

Several questions arise when one contemplates studies of breast cancer genetics, particularly as related to metastasis. How can one expect to define the genetic or biochemical basis of metastasis when it is clear that multiple genes and proteins are involved? Also, what can be done to compensate for the genomic instability associated with tumor progression? In other words, are we hunting a moving target? Are the same genes responsible for controlling metastasis in different histologic types of breast carcinoma (ie infiltrating ductal versus lobular carcinoma)? How does one identify metastasis-associated genes when environmental context is so important?

Historically, the experimental approach to answering these questions has been reductionist: mimic metastasis component steps (eg proliferation, adhesion, invasion, angiogenesis, evasion from immune cell killing, etc) *in vitro*, and study the gene(s) and protein(s) responsible for controlling each step. This approach has led to a tremendous understanding of fine molecular detail for each step, but translation to clinical utility has been limited for the reasons outlined below. Nonetheless, the genetic underpinnings are being elucidated, and fundamental biologic mechanisms behind the metastatic process are being unraveled.

Oncogenesis and tumor progression are related, but distinct, phenotypes

One area of major confusion regarding metastasis-associated genes has been the failure by some to recognize the important distinction between tumor formation and metastasis. Tumorigenesis and oncogenesis refer to a cell's ability to proliferate continuously in the absence of persistent stimulation by the triggering carcinogenic agent(s). Tumor progression is the evolution of already tumorigenic cells (populations) towards increasing malignancy. The distinction is crucial when considering whether a gene is important in controlling steps associated with malignancy as compared with whether that gene is involved in tumor formation.

The distinction between malignant and metastatic is more subtle. Pathologists characterize malignancy on the basis of several morphologic attributes, including less differentiated cytology, vascularity, necrosis, mitotic index, aneuploidy, and nuclear : cytoplasmic ratio. The incontrovertible hallmarks of malignancy are invasion of cells though a basement membrane and/or metastasis. All other characteristics used to label a tumor (and the cells within it) as malignant have exceptions [14]. For example, morphologically indolent cells may be behaviorally malignant and *vice versa*. Clearly, parameters associated with pathologic examination are invaluable when predicting the probability for local, regional, or distant recurrence in a clinical setting [15], but they are limited with regard to cause/effect relationships for genes.

In the context of a multistep, multigenic cascade, it is critical to recognize that the terms invasiveness and adhesion are not equivalent to metastatic propensity. Both invasion and adhesion are necessary, but not sufficient for metastasis. Cells that are efficient at either or both, but which lack the ability to complete any other step of the metastatic cascade, are nonmetastatic [16]. Therefore, correlations of genetic expression with a particular step in the metastatic cascade may lead to erroneous conclusions. This can occur in two directions. Inhibition of a step in metastasis, such as invasion, does not necessarily translate to complete inhibition of metastasis *in vivo*. Likewise, at least two recent papers [17••,18••] have demonstrated that nonmetastatic cells exhibit equal invasiveness (and a variety of other parameters) to their metastatic counterparts. The implication is simple; *in vitro* assays, as surrogates of metastasis, are not 100% predictive.

Taken together, these points emphasize the importance for distinguishing each of these phenotypes. Tumor-suppressor genes dominantly inhibit tumor formation when wild-type expression is restored in a neoplastic cell. By definition, then, metastasis should also be suppressed (because the cells are nontumorigenic). Metastasis-suppressor genes, on the other hand, block only the ability to form metastases. Restoring expression of a metastasis-suppressor gene would yield cells that are still tumorigenic, but that are no longer metastatic. From experimental and treatment perspectives, identification of suppressors of metastasis is much simpler than identifying metastasis-promoting genes. This is because it takes only one gene to block metastasis, whereas it takes the coordinated expression of multiple genes to allow metastasis. In experimental systems, it is fairly easy to find associations with metastatic ability, but it is difficult to prove that a particular gene is essential. For example, if one were to transfect a *bona fide* metastasis-promoting gene (ie one that promotes invasion) into a cell that already contains a defect in another gene (for instance, one that is required for angiogenesis), then the transfected cell would remain

nonmetastatic. In contrast, introduction of a gene that disrupts any step in the metastatic cascade would render cells nonmetastatic.

We recently reviewed the literature in breast cancer [19••] and found that differential expression of over 150 genes had been correlated with breast cancer development and/or progression. To date, however, only six human metastasis-suppressor genes have been demonstrated to have functional activity using *in vivo* metastasis assays: *NME1* [20•,21], *KiSS1* [22•,23], *KAI1* [24•,25], *CAD1* [26•,27], *BRMS1* [28], and *MKK4* [29••]. The following discussion summarizes the key information related to the discovery, activity, and mechanisms of action of these metastasis-suppressor genes.

Nm23

The first cloned metastasis-suppressor gene, *Nm23*, was identified in the murine K1735 melanoma using subtractive hybridization, because its expression was inversely correlated with lung colonization. Expression of the human homolog *Nm23-H1* (also known as *NME1*) is decreased in many, but not all late-stage, metastatic human cancers (for review [20•,30]). Decreased expression is the key parameter that determines metastatic potential, and may occur through a variety of mechanisms, not necessarily loss of heterozygosity [30]. The long-term prognostic value of this gene has been questioned in some studies [31,32]. Nonetheless, *NME1* is a *bona fide* metastasis-suppressor gene in human breast carcinoma, because transfection of metastatic MDA-MB-435 cells suppressed metastasis from an orthotopic site in an experimental mouse model [33]. Transfection into other cell lines has also resulted in metastasis suppression (for review [30]), including the human breast carcinoma cell line MDA-MB-435 and the rat mammary adenocarcinoma MTLn3. *In vitro* assays of control and *Nm23* transfectants have consistently pointed to decreased motility, invasion, and colonization.

The mechanism of action for Nm23 remains unknown. Nm23 is a member of the nucleoside diphosphate (NDP) kinase family of proteins [34]. NDP kinases are ubiquitous and catalyze the transfer of γ-phosphates, via a phosphohistidine intermediate, between nucleoside and deoxynucleoside triphosphates and diphosphates. However, NDP kinase activity can be dissociated from its metastasis-suppressor function [35,36]. Some recent reports suggest that *NME1* may control cell cycle progression [37], and histidine-dependent protein phosphorylation [38,39] and transcription [34,40]. The *Nm23* story becomes more complicated, because five additional family members have recently been identified and cloned (*Nm23-H2*/*NME2*, *Nm23-DR*, *Nm23-H4*, *Nm23-H5*, and *Nm23-H6*). Of these, only *NME2* has been tested for its role in metastasis, and the results are controversial [41–47].

KiSS1

Metastasis of human melanoma cell lines C8161 and MelJuSo is inhibited after introduction of an intact human chromosome 6, but tumorigenicity is unaffected [48,49]. *KiSS1* was cloned following subtractive hybridization that was performed to compare mRNA expression in chromosome 6-C8161 cells with that in parental C8161 cells. Preliminary data using cell lines indicates that *KiSS1* expression is lost as melanoma cells convert from radial to vertical growth phase (benign to malignant transformation) [22•], but more extensive clinical studies have been slowed due to lack of suitable antibodies.

Because *KiSS1* maps to chromosome 1q32 [22•,50] and because deletions and rearrangements of the long arm of chromosome 1 have been associated with breast cancer progression [19••], we tested whether *KiSS1* would suppress metastasis of the human breast ductal carcinoma cell line MDA-MB-435, which does not express *KiSS1* [51]. Transfection resulted in suppression of metastasis from the mammary fat pad of athymic mice, whereas vector-only transfectants were unaffected. Likewise, tumorigenicity was not suppressed.

The mechanism of action for *KiSS1* has not yet been determined, although its ability to suppress metastasis has been demonstrated in six independently derived human cancer cell lines of melanoma and breast origin [22•,23,51]. On the basis of the cDNA sequence, the predicted *KiSS1* protein is a hydrophilic, 164-amino-acid protein with molecular mass of 15.4 kDa. The sequence is novel, having no strong homology to any known human cDNA sequences. A recent report suggests that *KiSS1* may differentially regulate matrix metalloproteinases (MMP)s. Yan and Boyd [52] recently showed that *KiSS1*-transfected HT1080 cells showed specific downregulation of MMP9 transcription, whereas MMP2 transcription remained unchanged.

KAI1

Kai1 (also known as CD82 or C33) is a member of the tetraspanin superfamily of adhesion molecules, and *KAI1* was recently discovered to be a prostate cancer metastasis-suppressor gene, mapping to chromosome 11p11.2-p13 [53,54]. Kai1, like other members of the tetraspanin superfamily, has been associated with metastatic potential of nonsmall-cell human lung, liver, pancreatic bladder, breast, prostate, and esophageal carcinomas and melanomas (for review [55]). Downregulation of the *KAI1* gene is observed during the progression of human prostatic cancer, but mutations or allelic loss do not appear to be the major means for alteration [56]. Mechanisms in other tumor types have not been so extensively evaluated.

The role of *KAI1* in breast cancer metastasis has been implicated by several studies. *KAI1* mRNA expression progressively decreased in a panel of human cell lines

representing a continuum from nearly normal breast cells (MCF10A) to highly metastatic cells (MDA-MB-435) [57]. Transfection of *KAI1* into MDA-MB-435 cells suppressed metastasis from the mammary fat pad [25]. However, the cell lines did not maintain transgene expression levels following *in vivo* growth.

The mechanism of action of *KAI1* is not completely understood. Several preliminary reports suggest that expression of *KAI1* decreases the both the invasiveness and motility of cells *in vitro* [58,59]. These studies also showed that *KAI1* transfectants exhibited enhanced calcium-independent aggregation, suggesting that *KAI1* might alter cell–cell interactions.

CAD1

E-cadherin (encoded by the *CAD1* gene inhumans) is a cell-surface glycoprotein that is involved in calcium-dependent cell–cell adhesion. Reduced levels of E-cadherin are associated with decreased adhesion and increased grade of epithelial neoplasms, whereas increased E-cadherin expression (induced by transfection) decreases motility and invasiveness [60]. Mutations in E-cadherin and the associated protein α-catenin have been associated with acquisition of the invasive phenotype [61]. High E-cadherin levels inhibit shedding of tumor cells from the primary tumor, and thus E-cadherin is considered a metastasis-suppressor [26•,61–64]. However, there is also evidence that it can function as a tumor-suppressor gene [27,61,62].

A specific role for E-cadherin in breast cancer progression has not yet been established. However, mutations were detected, using polymerase chain reaction single-strand conformation polymorphism assays, in lobular carcinomas [65,66]. Interestingly, infiltrating ductal and medullary breast carcinomas showed few mutations. This highlights the point made above regarding grouping all tumors together.

It has even been suggested that E-cadherin function could be restored by treatment with tamoxifen [67], but whether this takes place in a clinical setting has not yet been explored to our knowledge.

BRMS1

Introduction of a normal, neo-tagged, human chromosome 11 into MDA-MB-435 cells suppressed metastasis without affecting tumorigenicity [68], leading to the hypothesis that a metastasis-suppressor gene(s) resides on chromosome 11. Differential display was performed in order to identify those genes and a novel gene, *BRMS1* (breast metastasis suppressor 1), was cloned [69••]. Transfection into MDA-MB-435 and MDA-MB-231 breast carcinoma cell lines suppressed metastasis without affecting tumorigenicity in a mouse model. The gene mapped to 11q13.1-q13.2, a region that is frequently altered in late-stage breast carcinoma [69••]. Following transfection, *BRMS1* restored gap junctional intercellular communication between cells, whereas vector-only transfectants still did not communicate in this manner. *BRMS1* transfectants were also significantly suppressed for motility *in vitro*. No data regarding expression or mutation patterns in human cancers yet exist.

MKK4

The introduction of a discontinuous, approximately 70-cM portion of human chromosome 17 significantly suppresses the metastatic ability of AT6.1 rat prostate cancer cells without affecting tumorigenicity [18••]. AT6.1 cells that contain the approximately 70-cM region escape from the primary tumor and arrest in the lung, but are growth inhibited unless the metastasis-suppressor region is lost [18••]. A combined differential expression and candidate gene approach identified the *MKK4/SEK1* (mitogen-activated protein kinase kinase 4/stress-activated protein/Erk kinase 1) gene as a candidate metastasis-suppressor gene that is located within the approximately 70-cM region [29••]. Transfection of an *MKK4/SEK1* expression construct significantly suppressed metastasis without affecting primary tumor growth. *In vivo* studies showed that AT6.1 cells that express the *MKK4/SEK1* transgene recapitulate the dormant phenotype conferred by the approximately 70-cM region of chromosome 17 [29••].

Previous studies had identified *MKK4/SEK1* as a candidate tumor-suppressor gene (for review [29••]). These studies identified homozygous deletions and other inactivating mutations in *MKK4/SEK1* in a small percentage of lung, pancreatic, and breast cancer cell lines and/or xenografts. Importantly, *MKK4/SEK1* can be an independent target for loss of heterozygosity (ie its inactivation is not just a byproduct of large deletions of the nearby *p53* gene). Recent studies using transgenic approaches (for review [29••]) found that disruption of the *MKK4/SEK1* gene caused embryonic death in mice, demonstrating a requirement for *MKK4/SEK1* in development. These studies also included the analyses of cells with a homozygous deficiency in *MKK4/SEK1*, and demonstrated that it is required for the normal regulation of cellular responses to environmental stress.

Colonization of the secondary site and future directions

The purpose of all of the research highlighted in this review has been to improve cure rates and patient quality of life. It has been argued that agents that prevent metastasis will be meaningless, because the 'horse will already have escaped the barn'. Administration of a preventive agent for an event that has occurred before diagnosis would indeed be useless. However, for inoperative lesions in an adjuvant setting, metastasis prevention may have a role.

Attention, then, turns to treatment of established metastases. In essence, all nonsurgical cancer therapy currently in use is essentially for this purpose. Once a tumor is removed, any additional treatment is aimed at eliminating microscopic or detectable systemic disease. What limits the current approaches is tumor heterogeneity, plasticity of the tumor cell response, and ineffective drug targeting.

How, then, will understanding the genetic basis of metastasis overcome these limitations? The answer is alluded to in some recent studies from our laboratories. In short, independently discovered metastasis suppressors for prostate carcinoma and melanoma both inhibit the formation of metastases by blocking growth at the secondary site [17••,18••]. In these studies, melanoma cells carrying chromosome 6 or rat prostatic carcinoma cells carrying chromosome 17 followed in spontaneous metastasis assays in mice. For instance, chromosome 17-expressing, tagged cells were found as microscopic metastases in the lungs at rates comparable to the number of detectable metastases produced by the metastatic parental cell line. The chromosome 17-expressing, tagged cells could be retrieved from the lungs and expanded in culture, demonstrating their vitality. No evidence was found for antiangiogenesis by the chromosome 17 hybrid cells, indicating a lack of colonization (ie not angiogenesis) as a primary mechanism. For the chromosome 6-expressing, tagged melanoma cells, *in vitro* explants of pulmonary micrometastases were injected into mice at an orthotopic site. The mice developed tumors that grew at a rate similar to that of the original cell line, further demonstrating that growth in the primary and secondary sites are, to some degree, differentially regulated. In other words, the metastasis-suppressed cells complete every step of the metastatic cascade before proliferation at the secondary site to form macroscopic metastases.

Although the existence of control mechanisms at this step of the metastatic cascade have long been inferred on the basis of logic, these data are the first hints at a molecular target. Neither of these genes could have been discovered without studying the entire metastatic process, because no *in vitro* assays yet recapitulate metastasis. Indeed, our laboratories are working hard to develop such assays. Nonetheless, these results show that *in vivo* assays still have a role and that novel, interesting, and potentially clinically relevant genes can be discovered by using them.

A second example of impaired colonization that is potentially applicable to growth at a secondary site is found in the *Nm23* literature. Colonization of *Nm23*-transfected K1735 melanoma and MDA-MB-435 human breast carcinoma cells in soft agar was reduced as compared with control transfectants, despite the observations among all transfection studies reported to date that primary tumor sizes are equivalent.

The cytokine transforming growth factor (TGF)-β has been reported to be inhibitory to cell growth of many normal cells, but recently has been widely reported to stimulate growth or colonization of more advanced or metastatically competent cells (for review [70,71]). Addition of TGF-β to soft agar cultures of control- and *Nm23*-transfected cells recapitulated this trend; TGF-β stimulated by several-fold the colonization of metastatic, control transfectants, but was generally without effect on the *Nm23* transfectants [33,72]. It is hypothesized that in a secondary site (where locally produced growth factors and cell–cell interactions may be different than those at the primary site) the cancer cells, which can utilize a widely available growth factor such as TGF-β as a stimulant, would have a metastatic advantage. Other cytokines, such as IL-6, have also been reported to exhibit a similar switch to the stimulation of aggressive cancer cells. The mechanism of the TGF-β 'switch' is unknown, but is of potential translational relevance. Investigations in other model systems have identified TGF-β-induced alterations in cell–cell interactions in the liver [73], production of antiapoptotic proteins [74], enhancement of proteinase activity [75,76], and induction of angiogenic factor production [77]. In bone metastasis, TGF-β is produced by osteoclasts and induces parathyroid hormone-related protein production by tumor cells in a positive feedback loop [78,79]. Other non-TGF-β-related studies of colonization also point to a myriad of potential control points. The most amazing aspect of this list is its overlap with the regulation of more traditionally studied aspects of metastasis: adhesion, proteolysis, and motility.

Colonization in various models has been influenced by adhesion proteins such as CD44 [80], α_6 integrins [81,82], galectin-3 [83], lung dipeptidyl peptidase [84], and N-cadherin [85], the latter pointing not only to adherence, but also to a cellular epithelial/mesenchymal transition [86]. The target of many adhesion processes, the stroma or extracellular matrix, is also reported in the colonization literature. Proteinases, such as MMPs and plasminogen activators, have been implicated in colonization, not only to include matrix degradation, but also for effects on tumor dormancy [87,88]. Growth factors and their receptors such as c-met and insulin-like growth factor receptor have been implicated in colonization [89,90]. Importantly, overexpression of fibroblast growth factor in MCF7 breast carcinoma cells facilitated dissemination from the primary tumor, but not lung colonization [91], showing that not any factor can be assigned to this phenotype. Potential colonization regulatory points outside of the traditional invasion arena include apoptosis and angiogenesis [92,93].

These data imply that whatever gene(s) and protein(s) are responsible could be exploited at two levels in the clinic. First, a mimetic could be used to prevent establishment of new metastases. This is demonstrated by the preclinical

studies performed using animal models. Second, a mimetic could block growth of the metastases and, perhaps, even cause the metastases to regress. The data also imply that many of the traditional components of metastasis research could be relevant to the study of colonization at the secondary site. They also highlight the need for better models.

The genetics of metastasis in general, and breast cancer specifically, is complex and still poorly understood. Although new genes/proteins are being identified at an increasingly rapid rate, a comprehensive and unifying model for interactions between them will require more research.

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