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Learning therapeutic lessons from metastasis suppressor proteins

Steven Christopher Smith and **Dan Theodorescu**
Department of Molecular Physiology and Biological Physics, and The Paul Mellon Urologic Cancer Institute University of Virginia Health System, Charlottesville, Virginia 22908, USA

Abstract

Metastasis suppressor proteins regulate multiple steps in the metastatic cascade, including cancer cell invasion, survival in the vascular and lymphatic circulation, and colonization of distant organ sites. Understanding the biology of metastasis suppressors provides valuable mechanistic insights that may translate to therapeutic opportunities. Several reports have explored novel strategies for restoring metastasis suppressor function, including gene transfer, induction of previously suppressed gene expression and exogenous administration of gene product. Pathways activated downstream of metastasis suppressor loss can also be targeted. Although none of these strategies are yet in routine clinical use, several are being tested preclinically and in clinical trials.

Myriad oncogenes and tumour suppressor genes have been functionally implicated in the process of transformation and tumorigenesis; these genes positively and negatively regulate the subsequent development of a primary tumour[1]. By contrast, a growing body of literature has defined another class of genes that function, positively or negatively, in the regulation of metastasis[2], the complex process through which malignant cancer cells leave a primary organ site, invade through basement membranes and connective tissue structures, journey to a distant site through the lymphatic or haematogenous circulation and finally establish a clinically detectable foothold in a distant organ[3]. Genes that regulate these steps in the metastatic cascade are similar to those that regulate transformation and tumorigenesis in the sense that they can be either promoters or suppressors of the phenotype. Just as tumour promoters such as oncogenic Ras or SRC and tumour suppressors such as PTEN or p53 regulate tumorigenesis, similar promoters and suppressors regulate metastasis.

For a few of these metastasis genes, loss of expression or function is requisite for the development of distant metastases, because they suppress one of the key steps of invasion, dissemination, arrest, survival and growth in a second parenchyma. Genes that inhibit

Correspondence to D.T. dt9d@virginia.edu.

DATABASES
ClinicalTrials.gov: http://clinicaltrials.gov/
NCT00134056|NCT00577122
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
SMAD4
National Cancer Institute Drug Dictionary: http://www.cancer.gov/drugdictionary/
atrasentan hydrochloride | dexamethasone | etoposide | medroxyprogesterone acetate | MPA
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BMP4 | BRMS1 | CD82 | DARC | DLC1 | ET1 | KISS1 | KISS1R | KSR1 | LPAR1| MAP2K4 | neuromedin U | NME1 | p53 | PEBP1 | PTEN | RAF1 | SRC
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metastasis but do not affect the ability of the transformed cells to produce a tumour at the primary site (which would define them as tumour suppressors) are known as metastasis suppressor genes. In this Review, we will provide perspective on these genes, discuss the rationale for targeting metastasis suppressor genes as a therapeutic modality, and review several cases in which such strategies have begun to show promise.

Perspective on metastasis suppressor genes

Until recently, few metastasis suppressor genes had been characterized: 5 years ago the list included only eight. In terms of broad ontology, these genes all had a similar function: regulating key cell signalling pathways, including both G-protein-coupled and tyrosine kinase receptor signalling, and small GTPase and MAPK signal transduction, reviewed recently[4]. However, in the ensuing few years the field has grown drastically, with one recent report reviewing more than 23 separate genes, including additional genes regulating key signalling pathways and genes regulating other functions as diverse as adhesion, migration, cell death and angiogenesis[5]. Further enriching the biological complexity, in 2008 an entire new class of microRNAs that suppress metastasis was described[6]. These molecules have been demonstrated to regulate metastasis through their ability to bind to the 3′ untranslated regions of and coordinately regulate key genes that mediate the metastatic phenotype[7]. Key milestones in the development of this field are summarized in the TIMELINE and recent reviews[5,8,9].

One reason that there have been relatively few metastasis suppressor genes described until recently is that their identification and characterization not only involves a convergence of several types of data, but also requires an *in vivo* metastasis model for testing suppressor function that models the natural history of the specific tumour type with reasonable fidelity. This has been greatly aided by the widespread availability of several immunocompromised mouse strains for xenograft models[10]. Experiments to identify metastasis suppressors often include screens to identify candidate genes by comparing cells or tissues of different metastatic competence, examining the expression or mutation status of such candidates in human tumour tissues and, indispensably, showing in an *in vivo* metastasis assay that reconstitution of the suppressor in fact does suppress metastasis formation without abrogating proliferation or tumorigenesis (BOX 1).

Box 1

The identification and characterization of metastasis suppressor genes

Screens of cells of differing metastatic properties to identify differentially expressed candidate genes

Strategies have used screens such as chromosome transfer to screen for metastasis suppressor loci[50], differential display and subtractive hybridization techniques[30], and comparative microarray studies of cell lines exhibiting differing *in vivo* metastatic potentials[90]. Recently, workup of candidate metastasis suppressor genes and targets has been aided by available microarray expression data for many tumour histologies[108], as candidates can be quickly examined across different stage tumours for expression patterns consistent with potential metastasis suppressor function[40,90].

Correlation of loss of candidate gene expression or function with development of metastasis in patients

Validation of a candidate metastasis suppressor gene should include examination of its expression or function in human tumour tissues, ideally from actual metastases. However, resection of such lesions is rare for most malignancies. Instead, surrogate measures of metastatic ability are used, including whether loss of expression of the candidate in the

primary tumour is associated with the development of metastasis in patient follow-up[92], clinicopathological surrogates of aggression (stage, grade and so on) or patient survival.

Demonstration of *in vivo* suppression of metastasis

Typically, control and derivative cells exogenously re-expressing a candidate metastasis suppressor are used to prove suppression of metastasis without hindering tumorigenesis, the *sine qua non* for defining a metastasis suppressor protein. These experiments generally use tumour-derived cell lines introduced into rodents[10]. Spontaneous metastasis assays examine cells in an orthotopic xenograft[109] or at subcutaneous or other sites in a heterotopic xenograft[110]. These evaluate the majority of the steps in the metastatic process, but not all tumour types have xenograft models amenable to this assay. Experimental metastasis assays involve injection of tumour cells directly into the arterial or venous circulation[111], examining only the latter portion of the metastatic process, but can be faster and more reproducible than spontaneous metastasis assays. Either way, effects on primary tumour growth must be ruled out by subcutaneous or orthotopic xenograft assays as well.

From function to therapeutic insights

The basic rationale for development of therapies targeting metastasis suppressor genes is the same as that for targeting metastasis in general[11,12]. However, mechanistic insights obtained from studying these proteins have led to the identification of what we believe is a key vulnerability of metastatic cancer and perhaps the most relevant target for therapeutic intervention: metastatic colonization at the second tissue site. Interestingly, circulating cancer cells or even those found as single or small clusters in distant organs are not necessarily the harbingers of clinically overt metastatic disease[13], although they are surprisingly pervasive[14,15] and seem to occur early in localized disease in both patient series[16,17] and animal models[18,19]. By contrast, colonization occurs when these disseminated cells grow from single or clusters of ectopic cancer cells (micrometastases) into clinically apparent macrometastases, a step that is extremely inefficient[20–22]. Even a US epidemiological survey speaks to the issue of the generality and relevance of this early seeding phenomenon in several of the most common cancer types. Steeg *et al*. examined the US National Cancer Institute SEER database, analysing tumour staging at diagnosis for several common cancers and found that, of cancer cases without clinically apparent metastases at diagnosis, between 29% and 37% of patients nonetheless exhibit regional lymph nodes that are positive for tumour cells[23].

In short, many tumours may have already disseminated widely on a cellular basis before the time of diagnosis[24]. Thus, as other authors have noted[25,26], in patients whose primary tumours are successfully treated, subsequent suppression of metastatic colonization is a salient therapeutic niche. This is a variation of the adjuvant therapy concept, which attempts to destroy cells in distant organs by delivering therapy following definitive treatment of the primary tumour. In the case of metastasis suppressor-targeted therapy, the same goal would be pursued, but by targeting the function of metastasis suppressor genes that work specifically at this key juncture rather than through conventional cytotoxic agents. Either way, this kind of suppression attempts to delay recurrence, which may equate to a functional cure in patients who die from non-cancer-related causes.

Though not all metastasis suppressor genes have been demonstrated to suppress this rate-limiting colonization step, a growing number of studies have demonstrated that several of them function at this point[27]; that is, in experimental assays modelling a setting of widely disseminated cells, their expression has been shown specifically to suppress single and small clusters of viable cells in a dormant state in the second parenchyma, whereas control cells progress to form gross macrometastatic nodules. The genes identified to date include KISS1, KAI1 (also known as CD82), NM23 (also known as NM23-H1 and NME1) and MAP2K4,

which have been reviewed recently[28,29], and others of the growing number of metastasis suppressors may also function at this stage. It also bears mentioning the caveat that these and other metastasis suppressor genes may also function at other steps in the metastatic cascade. Indeed, many have been shown experimentally to suppress processes such as cellular motility, invasion and survival during dissemination (FIG. 1). However, given the early, wide dissemination of cancer cells, these properties are likely to prove less adaptable to the colonization therapeutic niche.

The involvement of metastasis suppressors in the formation of macrometastases suggests that functionally reconstituting these proteins could be beneficial. In the case of several metastasis suppressors, recent key reports have developed strategies for reconstituting protein function that have already proved promising in pre-clinical studies (TABLE 1). These strategies fall into three broad categories, including reconstitution of metastasis suppressor expression by induction of the endogenous locus or by gene therapy (FIG. 2a–c), direct administration of the suppressor protein itself (FIG. 2d) and targeting essential downstream pathways that are activated by loss of suppressor function (FIG. 3). Below, we discuss representative studies that highlight these approaches as well as discuss future strategies that use advanced pharmacogenomic and bioinformatic approaches aimed at discovering agents that effectively target these key players in metastatic vulnerability.

Inducing expression as a therapeutic strategy

NM23

NME1 (non-metastatic cells 1), known in the field as *NM23*, was the first and most extensively described metastasis suppressor gene. It was discovered in 1988 by Steeg and associates using differential hybridization experiments comparing gene expression between high- and low-metastatic cell lines derived from the K-1735 murine melanoma cell line[30]. They identified *NM23*, the expression of which could abrogate spontaneous and experimental metastasis without affecting tumour cell growth *in vitro* or *in vivo*[31]. Subsequent studies have identified up to eight homologues in the human genome, of which *NM23-H1* (herein *NM23*) and *NM23-H2* (*NME2*) have been shown to function in metastasis suppression[32]. These findings have been confirmed by others in different experimental systems and cell types[33–35].

NM23 has several interesting properties[32]. First, it exhibits histidine kinase activity toward several proteins, including KSR1, the kinase suppressor of Ras, resulting in increased KSR1 degradation and decreased MAPK signalling[36], which has been shown in many tumour types to support survival, motility and other cellular processes that are important for metastasis[37]. NM23 activity may also be modulated *in vivo* by differential binding to endogenous and exogenous viral proteins[32], including the Epstein–Barr virus (EBV) nuclear antigens 1 and 3C. This results in relief of NM23-dependent suppression of migration[38] and metastasis[39], suggesting that its function can be abrogated without loss of endogenous expression[32]. Finally, NM23 suppresses metastasis by reducing the expression of pro-metastatic genes[40] (see the section ‘Targets downstream of metastasis suppressors’).

Therapeutic exploitation of NM23 function is based on studies of its promoter[41], which is regulated by the glucocorticoid response pathway. Although addition of the glucocorticoid receptor agonist dexamethasone could not increase NM23 expression pharmacologically, the progesterone and atypical glucocorticoid agonist medroxyprogesterone acetate (MPA) induced expression in a glucocorticoid receptor response element-dependent manner[42]. MPA, commonly used at low doses for contraception, has previously been used at higher doses in breast cancer clinical trials[43,44]. Preclinical evaluation of MPA as an inhibitor of metastasis formation revealed promising results. Four weeks after injection of a reliably metastatic sub-line of MDA-MB-231 breast cancer cells into nude mice, lung micrometastases had formed.

The animals were then randomized to either control or MPA treatment regimens that approximate the serum MPA concentrations that are achievable in human patients. Significant decreases in the proportions of animals developing clinically relevant metastases (decreased by 27% and 36% in two separate experiments) and average disease burden per animal were noted after treatment with MPA and, in animals that failed treatment, the proportion of metastases staining more strongly for NM23 by immunohistochemistry was significantly higher than that of untreated mice[45]. On the basis of these promising results a Phase II trial has been initiated that is examining the clinical utility of MPA with and without anti-angiogenic low-dose, frequent cyclophosphamide–methotrexate metronomic chemotherapy[46] in refractory, hormone receptor–negative metastatic breast cancer (NCT00577122). NM23 has also been reported to be re-induced by treatment with retinoic acid[47] and oestradiol[48].

Restoration of NM23 function has also been attempted using adeno-associated virus-mediated gene therapy in a mouse model of metastatic ovarian cancer that used an aggressive variant of the SW626 cell line selected *in vivo*. After orthotopic injection of these cells to form a xenograft ovarian tumour, animals were treated by intraperitoneal injection of virus engineered to express NM23, which significantly decreased liver metastases (by 60%, $p = 0.01$) and increased the median time of survival compared with animals treated with control vector (by 35 days, $p < 0.05$)[49].

KAI1

Re-induction of endogenous metastasis suppressor function has also been reported for the metastasis suppressor gene *KAI1*. The activity of KAI1 was identified initially by transferring human chromosome 11 into AT6.1 rat prostate cancer cells[50,51], with subsequent cloning of the suppressing locus and observation of suppression of metastasis *in vivo*[52]. Two key suppressor functions that have been described for this membrane-bound protein are downregulation of epidermal growth factor receptor signalling, which is associated with increased receptor desensitization and endocytosis[53], and a novel pathway involving KAI1 and the Duffy antigen receptor for chemokines (DARC), which is expressed on endothelial cells. Circulating KAI1-expressing cells attach to endothelium through a KAI1–DARC interaction[54]. This interaction is associated with cessation of proliferation and induction of senescence in KAI1-expressing disseminated cells but not in those that do not express it.

As with *NM23*, transcriptional regulation of *KAI1* suggested several strategies for re-expression of this protein. A key early study found that p53 increased transcription of *KAI1* through a p53-responsive element[55]. The clinical relevance of this pathway was demonstrated by immunohistochemical staining of prostate cancer tissue specimens for p53 and KAI1, showing a significant concordance of expression. Proceeding from this observation, Mashimo *et al.* used etoposide, an agent that induces p53, and found that it induced KAI1 expression[56], suggesting that it might provide a therapeutic angle. Wu *et al.* treated mice with etoposide after splenic injection of gastric cancer cells and observed both induction of KAI1 expression in subsequent splenic tumours and reduced incidence of hepatic metastases[57], although this anti-metastatic effect was not proved to be specific to KAI1 function. It is tempting to speculate that etoposide treatment in patients could therapeutically induce KAI1 expression and, given the ease of oral administration, might be adaptable to a long-term suppression strategy, such as low-dose metronomic chemotherapy[58], a therapeutic setting in which it has been used previously[59]. However, given the pleiotropic effects of etoposide and p53 induction, the contribution of KAI1 induction might be minor and requires careful elucidation.

Another potential therapeutic strategy for long-term re-expression of endogenous KAI1 in prostate cancer is through the use of the soy-derived phyto-oestrogen isoflavone genistein. El Touny *et al.* recently reported reversal of loss of prostatic KAI1 expression in the TRAMP mouse model of prostate cancer following treatment with genistein[60]. *In vitro*, genistein

treatment inhibited the invasive behaviour of tumour-derived cells from this model. This anti-invasive effect of genistein was shown to be specifically due to KAI1 induction — rather than to any of the many other genes that are probably affected by genistein treatment — by demonstration of reversion to an invasive phenotype when the induced KAI1 was depleted by small interfering RNA. However, the impact of this approach on the metastasis of the TRAMP tumours was not examined in this preclinical study, and the mechanism of KAI1 induction remains unknown.

Traditional gene therapy techniques have also been used to increase the expression of KAI1. Xu *et al.* compared direct injection of saline with either a KAI1 plasmid expression vector or liposomes containing the expression vector into xenografted MiaPaCa II pancreatic cancer cells established as subcutaneous tumours in nude mice. Interestingly, injection of the vector encoding *KAI1* either alone or in liposomes substantially reduced spontaneous pulmonary metastasis[61]. Another study used a replication-deficient adenovirus to reconstitute KAI1 expression in an orthotopic non-small-cell lung cancer lymphatic metastasis mouse model[62]. In this study, Lewis lung carcinoma cells were directly injected into the lungs of syngeneic mice to establish tumours. Mice were treated three times by intratracheal administration of viral particles engineered to express either control lacZ or KAI1, and lung and mediastinal lymph node weights were evaluated after 3 weeks. Treatment with KAI1-expressing virus, verified by immunohistochemical evaluation of expression in tumours at necropsy, was associated with a significantly decreased weight of mediastinal metastases but not with decreased primary tumour volume.

RKIP

Finally, Raf kinase inhibitor protein (RKIP, also known as PEBP1) was recently determined to function as a suppressor of spontaneous metastasis of orthotopically implanted C4-2B human prostate cancer cells in mice[63]. RKIP was first identified by a yeast two-hybrid screen for proteins that bind the RAF1 kinase domain and was shown to competitively inhibit RAF1–MEK interaction and downstream signalling[64]. RKIP is lost in prostate and other tumour types[65,66], and recent reports have suggested possible modalities for re-induction of endogenous RKIP expression.

Endogenous RKIP has been shown to be induced by treatment of cells with trichostatin A[67], a histone deacetylase inhibitor, suggesting that this class of drugs, which is under evaluation for cancer treatment[68], could potentially be used to induce RKIP expression. This highlights the potential use of chromatin-modifying drugs for therapeutic re-induction of silenced metastasis suppressor expression. Although relatively non-specific — that is, resulting in induction of many suppressed loci — this strategy has the potential to function for multiple metastasis suppressor genes, as has been shown for DLC1, another recently described metastasis suppressor[69]. Continuing with the theme of the use of drugs to de-repress metastasis suppressor genes, NDRG1 (REF. [70]) has recently been reported to be induced by the DNA methyltransferase inhibitor 5-azacytidine as has NM23 (REF. [71]).

Perspective

As a general therapeutic strategy, re-expression of an endogenous suppressor (FIG. 2a,b) is straightforward and appealing, especially when achieved with orally bioavailable agents. However, to date, these modalities depend on targets that have been transcriptionally downregulated instead of lost by mutagenesis or other mechanisms. Although some reports suggest that reduced transcription, rather than mutation, explains a large part of the loss of metastasis suppressor gene expression observed in different tumour types[72–75], isolated reports do observe mutations[76,77]. In fact, the issue of restoration of endogenous expression highlights one of the potential advantages of targeting metastasis suppressors over related tumour

suppressor genes: tumour suppressors are often inactivated by mutation early in the development of cancer and therefore would not be amenable to re-induction at the endogenous locus. The mechanism of loss must be established on a gene-by-gene, patient-by-patient basis in order to correctly identify and target these approaches in the clinic. As it is unlikely that most metastasis suppressor genes can be induced by agents as well tolerated as, for example, MPA, a concerted effort to discover orally bioavailable agents that can transcriptionally induce metastasis suppressors is warranted. Thus, despite the recognized difficulties of exogenous cancer gene therapy[78] (FIG. 2c), such an approach can be generalized to different target genes and may prove necessary for suppressors that cannot be easily re-expressed from the endogenous locus.

Direct administration of metastasis suppressor

KISS1

Administration of the metastasis suppressor protein KISS1 has shown preclinical utility in attenuating metastasis in tumours that have lost KISS1 function. *KISS1* was identified by subtractive hybridization between the human melanoma cell line C8161 and a derivative into which human chromosome 6 was transferred[79]. This chromosome is frequently lost in metastatic melanoma[80] and can suppress metastasis in these cells. KISS1 is located on chromosome 1 but is regulated by *trans*-acting factors on chromosome 6. *KISS1* encodes a protein that is modified into a secreted kisspeptin known as metastin, which agonizes at least one G-protein-coupled receptor known as KISS1R or GPR54 (REF. [81]). Although downstream signalling events leading to metastasis suppression have yet to be elucidated, one report demonstrates that secretion of KISS1 is necessary for metastasis suppression and that secreted KISS1 maintains dormancy in disseminated cells, thus blocking metastatic colonization[82].

In a preclinical study, Ohtaki *et al.* showed that spontaneous pulmonary metastasis of KISS1R-overexpressing B16-BL6 melanoma cells was significantly suppressed by administration of KISS1-derived peptide through an osmotic pump (by 66% ($p < 0.01$) compared with vehicle-treated controls)[83]. They hypothesized that KISS1 could be similarly administered to patients to maintain metastatic dormancy in a clinical setting. Recent reports have questioned whether the metastasis-suppressing effect of KISS1 is due to ligation of KISS1R on the cancer cell itself, a paracrine effect on neighbouring stroma or its effect on some other uncharacterized receptor[82]. However, this strategy seems promising. Another recent publication described development of small molecule mimetics of KISS1 (REF. [84]), suggesting another potential angle for administration of KISS1. Although administration of a KISS1 peptide to patients has been undertaken and tolerated in the short term[85], at least one preclinical animal model has revealed effects on the pituitary–gonadal axis, suggesting the possibility of long-term toxicity to male subjects[86]. Certainly any small molecule mimetic would require a similar careful preclinical and clinical trial workup. These issues must be clarified if this approach is to be successfully used in humans as it is likely that delivery of this protein would be required on a chronic basis, given the paradigm of suppression of the micrometastasis to macrometastasis transition.

BMP4

Just as KISS1 is a secreted factor, BMP4 (bone morphogenic protein 4), a member of the transforming growth factor-β superfamily of growth factors, has recently been reported to be a metastasis suppressor. Eckhardt *et al.* recently observed an association of reduced expression of BMP4 with increased metastatic potential in mouse mammary tumours[87]. They demonstrated that overexpression of BMP4 in 4T1.2 mouse breast cancer cells suppressed the formation of spontaneous metastases but not tumorigenesis, whereas stable depletion of BMP4 by RNA interference increased metastatic potential in less metastatic cell lines. Finally, they

observed that intraperitoneal administration of recombinant BMP4 suppressed spontaneous metastasis and enhanced survival in a preclinical model of breast cancer metastasis (R. Anderson, unpublished data, also presented at the 2008 Meeting of the American Association for Cancer Research–Metastasis Research Society, Vancouver, Canada).

In general, it is unlikely that administration of a recombinant metastasis suppressor protein (FIG. 2d) would be applicable to many metastasis suppressor genes as most are transmembrane or intracellular proteins. Indeed, only one other secreted metastasis suppressor protein has been described: CTGF (connective tissue growth factor)[88]. However, these cases of KISS1 and BMP4 provide a promising, although preliminary, foundation for such a strategy.

Targets downstream of metastasis suppressors

Several metastasis suppressor genes modulate the cellular signalling of cancer cells[4], which raises the question of whether the expression of downstream metastasis suppressor-regulated transcripts contribute significantly to the invasive and metastatic phenotype and whether these can be targeted therapeutically. Two recent reports suggest that this may be the case and have yielded druggable targets with clinical potential.

RHOGDI2

RHOGDI2 (RhoGTPase dissociation inhibitor 2, also known as ARHGDIB), a member of the RHOGDI family of proteins that downregulate the activation state of Rho family GTPases[89], was initially identified by our group as a metastasis suppressor in human bladder cancer by comparison of transcriptional profiles of the T24 bladder cancer cell line with a metastatic derivative, T24T[90,91]. Candidate genes differentially expressed between these two cells were screened against a panel of microarray data from multiple human tumour types for a pattern of decreased expression as a function of stage and grade. This screen yielded RHOGDI2, which suppresses experimental pulmonary metastasis of T24T cells without affecting tumour growth rate. Moreover, we have observed that loss of expression of RHOGDI2 is associated with poor survival outcomes in bladder cancer patients[92], whereas others have found that loss of RHOGDI2 is associated with nodal metastasis in breast cancer patients[93] and is part of a gene expression signature of metastasis in breast tumours[94].

Hypothesizing that RHOGDI2 loss leads to deregulation of signalling pathways and that resultant changes in transcription are necessary for metastasis, our group[95] profiled gene expression of RHOGDI2-transfected T24T cells and control cells (FIG. 3a). Transcripts that were downregulated in RHOGDI2-transfected T24T cells, but also overexpressed as a function of stage in human bladder cancer, were selected for further study. This screen yielded ET1 (endothelin 1), a potent vasoconstrictor, as a candidate gene whose effect on the endothelin A receptor may be antagonized by atrasentan hydrochloride. It bears note that for reasons unrelated to its connection to RHOGDI2, atrasentan is currently in Phase III trials for stage IV prostate cancer (NCT00134056). Administration of atrasentan to mice injected intravenously with T24T cells reduced the proportion of animals developing metastases (53% control versus 5% atrasentan treated, $p < 0.0001$)[95]. As atrasentan is orally bioavailable and has shown activity in prostate cancer[96] and pulmonary hypertension[97], it would seem to be an ideal candidate for use as adjuvant therapy for patients at high risk of metastasis development after radical treatment of their primary tumour. Interestingly, RHOGDI2 also suppresses the expression of neuromedin U, a molecule with many similarities to ET1, which mediates both increased growth of metastases and increased tumour cachexia in animal models[98]. This again reinforces the fact that gene expression regulated by metastasis suppressors can mediate key aspects of this disease. Unfortunately, agents that block the neuromedin U receptor are not available, although development of such a product would provide another way to target key players downstream of loss of RHOGDI2.

NM23

A similar approach[40] was used to discover NM23-regulated transcripts in MDA-MB-435 breast cancer cells (it is worth noting that significant controversy exists regarding whether MDA-MB-435 cells are derived from breast cancer or melanoma[99]). Candidate genes were identified by differential expression between control and *NM23*-transfected cells (FIG. 3b), and inverse correlation between candidate expression and NM23 expression in microarray data from two cohorts of human breast cancer patients was used to further winnow candidates. The lysophosphatidic acid receptor, LPAR1 (also known as EDG2), emerged from these analyses, and when exogenously expressed in breast cancer cells it rescued NM23-suppressed cellular motility[40]. This suggested a model in which loss of NM23 expression induces LPAR1 expression and increases cellular motility in cancer cells. As motility is only part of the metastatic process, the same group examined the *in vivo* metastatic properties of NM23 and LPAR1 co-expressing cells to see whether this system was relevant to lung metastasis. They found not only that LPAR1 expression enhances pulmonary retention of MDA-MB-435 cells injected intravenously in an experimental metastasis assay, but also that its co-expression with NM23 rescues NM23-suppressed pulmonary metastasis in these cells[100]. These findings immediately suggest a translational angle using antagonism of LPAR1 by Ki16425, an Edg family antagonist[101], for which preclinical metastasis studies are ongoing[102].

Future strategies and emerging technologies

The aforementioned identification of downstream targets regulated by loss of metastasis suppressor function depends heavily on microarray studies of gene expression patterns. Despite the serendipity of ET1 and LPAR1 being druggable targets, such a strategy is unlikely to work in every case. However, at a minimum every case should provide a signature of the gene expression changes induced by metastasis suppressor re-expression owing to the reversion of the cell to a non-metastatic phenotype. Intriguingly, the recent development of the Connectivity Map[103] provides a novel technology for systematically targeting such a signature.

The Connectivity Map is a massive repository of gene expression data from several cell lines after treatment with >1,000 bioactive compounds, providing the signatures of gene expression induced by such compounds. Searching gene expression signatures induced by metastasis suppressor re-expression against these drug-induced signatures provides a systematic framework for the potential discovery of drugs that target an entire signature, avoiding the problem of finding individual drug-gable targets such as ET1 or LPAR1. Such a strategy might also be more potent owing to its causing the reversion of several genes that contribute to the metastatic phenotype. Advantageously, such a signature-based therapeutic approach provides a built-in, specific signature to identify cases in which such a drug is likely to work, providing a biomarker that can select patients most likely to benefit, one of the hallmarks of personalized cancer therapy.

Recently, new informatic techniques have been developed to extrapolate drug efficacy from cell line model systems to patient outcomes[104,105]. These techniques use microarray studies of cell line gene expression and *in vitro* chemotherapeutic sensitivities to generate robust and parsimonious signatures predicting drug sensitivity in cell lines, then test these signatures against independent cell lines sets or, most compellingly, outcomes of clinical trials where patient tumours were profiled with microarrays before treatment. One of these approaches, the COXEN (Coexpression Extrapolation) system developed in our group[104], is adaptable to drug discovery and, in particular, to the setting of metastasis suppressor-dependent transcriptional deregulation. COXEN is an algorithm that uses data from microarray analyses and empirically determined drug sensitivities from the NCI-60 screen of cancer cell lines[99] to discover genes of which expression is associated with drug sensitivity. Then a specialized algorithm compares the expression of this sensitivity-related gene set from the cell lines to the same genes from

human tumours, identifying a subset of these genes whose correlated co-expression is relevant to the human tissue data and resulting in selection of a sensitivity prediction signature that works for human tumours in patients, as has been reported[104].

Importantly, COXEN can also facilitate computational drug discovery to identify agents or agent combinations that are highly effective in human patients based on the *in vitro* effects of these compounds on these cell lines. Thus, the Connectivity Map could use microarray signatures of cells with and without re-expression of metastasis suppressors to generate a list of drugs that induce the signature, and screening such drugs through COXEN and the NCI-60 screen could determine which agents both additionally show efficacy *in vitro* and have a high likelihood of working on patients (FIG. 4).

Another recent key development in drug discovery, which is imminently applicable to targeting metastasis suppressor genes, is that of synthetic lethal screens[106]. Conceptually adapted from yeast biology, these screens rely upon the premise that cells lacking a specific protein (such as a metastasis suppressor) are more sensitive to specific drugs than cells that have the protein. Proof of principle of this technology has been reported for *SMAD4*, the pancreatic tumour suppressor gene[107]. Wang *et al*. used a pharmacological synthetic lethal strategy to screen combinatorial pharmaceutical libraries against isogenic cell lines that differ solely in exogenous expression of (and, complementarily, RNA interference depletion of) SMAD4. Such a strategy yields candidate compounds that are selectively toxic to a cell that lacks suppressor function, whether by downregulated expression, mutation or otherwise. Such approaches are probably feasible for metastasis suppressors as well.

Summary

In summary, metastasis suppressor genes define a relatively small group of proteins, of which expression or function is lost in cancer cells as metastatic competence increases. When reconstituted in model systems, they suppress *in vivo* metastasis without abrogating tumour growth. Experimental and epidemiological data suggest that tumours shed many cells into circulation by the time of patient diagnosis. Some of these die in the circulation or after attachment in distant organs, whereas others survive and either remain dormant as a micrometastasis or grow to colonize the organ, forming a clinical macrometastasis. Therefore, suppressing the micrometastasis-to-macrometastasis transition, which is the point at which many metastasis suppressors function, should provide a promising therapeutic window.

Although targeting a negative regulator is technically challenging, recent reports have successfully reinduced expression, administered the gene product itself or targeted druggable molecules regulated by the suppressor gene. As functional genomic and proteomic technologies increase the speed and ease of discovery of new metastasis suppressors, the field of targets is likely to swell both in number and kind, particularly given the recent description of microRNA metastasis suppressors. As the field grows, and additional novel strategies for therapeutic intervention are developed, unanswered questions will become more salient. Among others, questions of generality (the relevance of a particular metastasis suppressor to different types of cancer), specificity (the relevance of a particular metastasis suppressor to suppression of metastasis in different second sites) and toxicity to normal cells (BOX 2) will need to be addressed in a systematic manner. Future candidate metastasis suppressor genes may prove more or less tractable as targets. Nonetheless, the strategies described herein form a framework for transposing these promising findings to other genes, and novel informatic technologies might ease drug discovery and patient selection for future metastasis suppressor-based therapies.

Box 2

Key unresolved questions in metastasis suppressor research

Generality

The relevance of most described metastasis suppressors across various types of cancer has only been superficially validated in pathological studies; rates of loss of expression and mutation, as appropriate to therapeutic strategies, must be carefully and independently validated. Given observations of early, wide cellular dissemination of tumour cells[24], are metastasis suppressors that chiefly inhibit invasion the relevant targets?

Specificity

Recent reports suggest rich complexity in the genetic regulation of organ-specific tropism of metastasis[112,113]. It follows that metastasis suppressor function might also be highly organ specific. The ability of any given metastasis suppressor gene to suppress metastases to multiple organs has only just begun to be assessed, with two recent reports examining the role of metastasis suppressors in metastasis to multiple organs[82,114].

Adaptability

As a corollary to the above questions, is it true that a strategy reconstituting a metastasis suppressor should necessarily only work in cancers where the suppressor or its function is commonly lost? Might its reconstitution in ‘irrelevant’ cancer types still target latent pathways resulting in the same goal of suppression of metastasis?

Toxicity

One of the theoretical advantages of reconstituting a lost, negative regulator such as a suppressor is that toxicity is likely to be lower than when poisoning a positive regulator that operates physiologically in normal cells. Has the observation that several metastasis suppressors impinge directly and negatively on essential cell signalling pathways[4] called this postulate into question?

Integration

The integration of metastasis suppressor-targeting therapeutics into current therapeutic regimens is non-trivial: would therapy resulting in suppression of distant proliferation of micrometastases not decrease sensitivity to traditional cytotoxic chemotherapeutics?

Patient selection

Several targeted agents now available for cancer therapy have been shown to be highly efficacious in patient subsets exhibiting particular molecular lesions. What biomarkers would be used for individualized therapy targeting metastasis suppressor genes? Is a primary tumour the appropriate tissue to analyse?

Trial design

Patients who enter clinical trials often have failed standard therapies and developed gross metastatic recurrences. Could such candidates even potentially benefit from metastasis suppression therapies?

At a glance

- Metastasis accounts for the preponderance of morbidity and mortality in cancer, and accumulating evidence suggests that dissemination of tumour cells may occur earlier in the development of cancer than previously appreciated.

- Metastasis is regulated either positively or negatively by proteins that promote steps in the metastatic cascade or those that suppress them.
- Metastasis suppressor genes encode proteins that prevent or reduce the development of metastases *in vivo*, without simultaneously affecting primary tumour growth. This is in contrast to tumour suppressors, which affect primary tumorigenesis. Metastasis suppressor proteins are lost primarily during cancer progression and not during transformation.
- Until recently, relatively few metastasis suppressor genes had been characterized. However, expanding genomic technology has made possible in recent years the description of many metastasis suppressor genes impinging on a wide variety of cellular processes.
- Although metastasis suppressor genes have been shown to work at multiple steps in the process of metastasis, several have been recently demonstrated to specifically suppress the colonization step of metastasis, the process by which solitary or small clusters of tumour cells living in a second organ site (micrometastases) grow to form clinically apparent and lethal macrometastases.
- Recent work has shown techniques that restore metastasis suppressor function. These include re-expression of the gene from the endogenous locus or by exogenous gene therapy, direct administration of the protein itself and targeting important metastasis mediators downstream of the suppressor that are reciprocally induced with metastasis suppressor protein losses.

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Glossary

Metastatic colonization	The process by which disseminated tumour cells, present as single or small clusters of cells in a second parenchyma (micrometastasis), grow to form a clinically detectable metastatic nodule (macrometastasis)
Epstein–Barr virus	(EBV). EBV is a herpes family virus that commonly causes infectious mononucleosis in humans. Infection with EBV has been implicated in Burkitt's lymphoma and nasopharyngeal carcinoma, and may also be involved in the pathogenesis of other tumours
Glucocorticoid response pathway	This nuclear hormone receptor pathway directs the regulation of genes involved in metabolism and immune function. Transcription by the glucocorticoid receptor is ligand-induced by glucocorticoid steroid hormones
Metronomic chemotherapy	Administration of chemotherapeutic drugs at comparatively low doses on a frequent or continuous schedule, with no extended interruptions, in contrast to traditional maximum tolerated dose chemotherapy
TRAMP	An autochthonous transgenic mouse model of prostate cancer. Various stages of progressive prostate disease can be observed in

	TRAMP mice, with focal adenocarcinomas developing between 10 and 20 weeks of age with 100% frequency
Mediastinal lymph node	Lymphatic tissue occurring in a central region of the chest between the lungs and bordered by the thoracic inlet above and the diaphragm below, where lung and other cancers frequently metastasize
Orthotopic xenograft	Establishment of a tumour by injecting human cells into the same rodent organ from which the human tumour was derived
Heterotopic xenograft	Establishment of a tumour by injecting human cells into a different rodent organ than that from which the human tumour was derived, often for technical reasons
Histone deacetylases	Enzymes that regulate chromatin structure and function through the removal of the acetyl group from the lysine residues of core nucleosomal histones, generally repressing transcription
DNA methyltransferases	Enzymes that catalyse transfer of a methyl group to cytosines in DNA, generally repressing transcription
Anoikis	Cell death in response to loss of matrix attachments
Osmotic pump	Generally small implantable pumps that use a salt concentration gradient to draw in interstitial fluid and generate pressure to expel a drug in a regulated fashion
Synthetic lethal	Describes a genetic phenomenon in which the combination of two otherwise non-lethal mutations or molecular lesions results in an unviable cell. In the case of drug discovery, the pharmaceutical agent, otherwise non-toxic, substitutes for one of these lesions and becomes lethal only in the presence of the second molecular lesion

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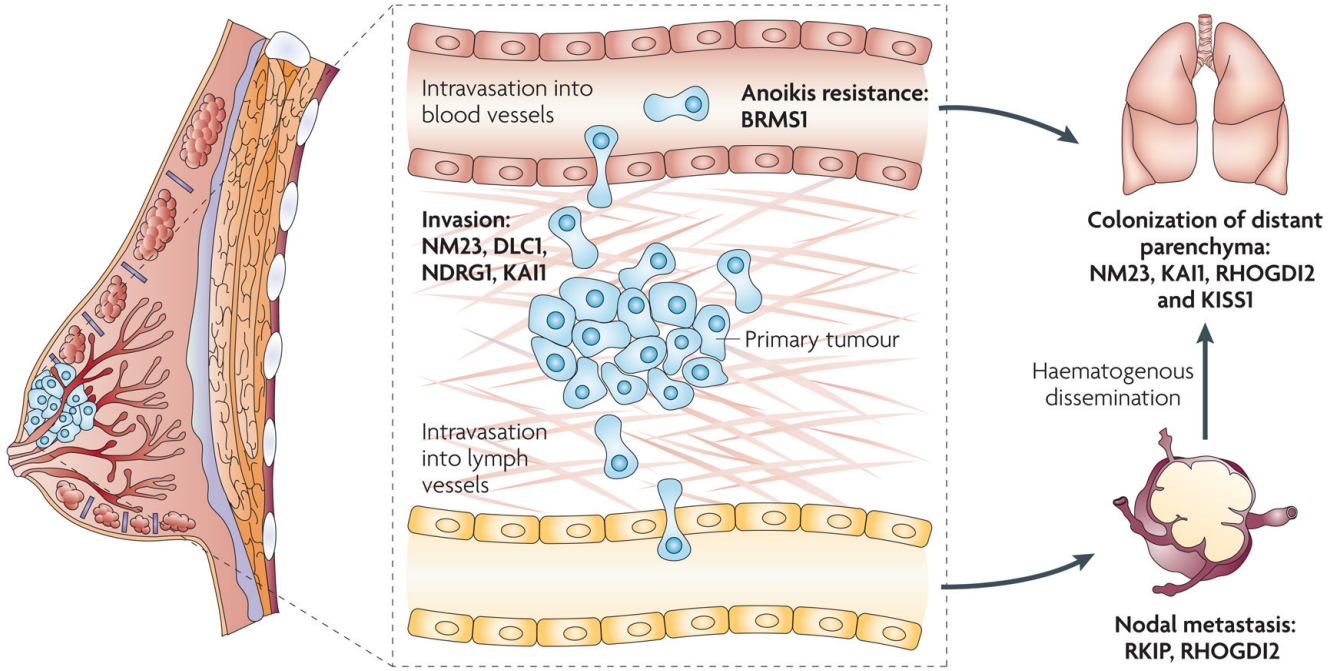


Figure 1. Metastasis suppressor genes and steps in the metastatic cascade in human cancer

The biological process of metastasis is a complex cascade with multiple steps in which suppressor activity may prevent clinically apparent metastasis. Given the prevalence and mortality of human breast cancer, we focus here on this tissue type, although these principles apply to most solid malignancies. In the primary tumour, deregulation of oncogenes and tumour suppressor genes mediates the conversion of normal cells to a neoplastic phenotype[1]. By definition, metastasis suppressor genes do not prevent these steps, but must function subsequently in the cascade as shown. Invasion of the basement membrane, stroma and vasculature constitutes one key, negative prognostic turning point in the natural history of breast and other cancer types. Many metastasis suppressor genes, including NM23 (REF. [40]), DLC1 (REF. [115]), KAI1 (REF. [116]) and NDRG1 (REF. [70]) have been shown to function in *in vitro* and *in vivo* surrogates of invasion in breast cancer cells. Nodal metastasis is also a key prognostic stage for breast cancer patients[117], now aggressively managed clinically. Loss of the metastasis suppressors Raf kinase inhibitor protein (RKIP)[66] and RhoGTPase dissociation inhibitor 2 (RHOGDI2)[93] have been shown to be associated with nodal metastasis in breast cancer. Survival during circulatory dissemination is another step of the metastatic cascade that has been studied in less detail; however, several reports have suggested that resistance to anoikis is important for metastasis[118] and that the metastasis suppressor gene BRMS1 (breast cancer metastasis suppressor 1) increases anoikis[114,119]. Because increasing data suggest that tumours are widely disseminated on an individual cellular basis even at the time of diagnosis of localized disease, the role of metastasis suppressors in preventing outgrowth of isolated single or cellular clusters (micrometastases), known as the ‘colonization stage’ of metastasis, is compelling. NM23 (REF. [29]), KAI1 (REF. [54]), RHOGDI2 (REF. [94]) and KISS1 (REFs [82], [120]) have been shown to function at this stage.

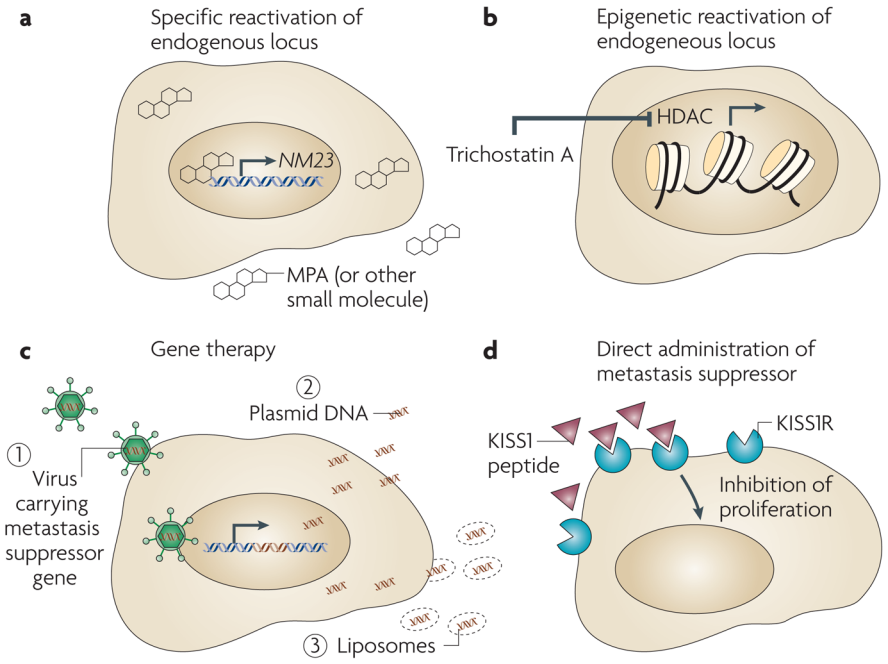


Figure 2. Strategies for restoring metastasis suppressor function

Given the key roles shown for metastasis suppressors *in vivo* and the association of their loss with negative outcomes in patients, re-expression of these proteins would seem a rational therapeutic strategy. This has been accomplished by strategies that are both specific — for example, re-induction of endogenous NM23 expression by medroxyprogesterone acetate (MPA)[45] (**a**) — and general — for example, the use of chromatin-modifying drugs[67,70,71] (**b**), including trichostatin A, which induces repressed gene expression by inhibiting histone deacetylases (HDACs). Exogenous viral gene therapy for re-expression of metastasis suppressor protein has also been reported (**c**) and has shown promise in preclinical models[49,61,62]. Non-viral vectors[121] for suppressor re-expression, including naked plasmid and cationic liposomes, have also been reported[61]. Direct administration of the metastasis suppressor protein is another strategy (**d**). In at least two cases, the metastasis suppressor protein itself is a soluble, secreted factor, amenable to direct therapeutic administration, in a fashion analogous to that of insulin. In 2001, Ohtaki *et al.* reported the use of osmotic pumps to administer KISS1 to prevent the development of metastasis[83]. This may proceed through induction of dormancy through KISS1R or another receptor[82] and, for KISS1, small molecule mimetics are also under development[84].

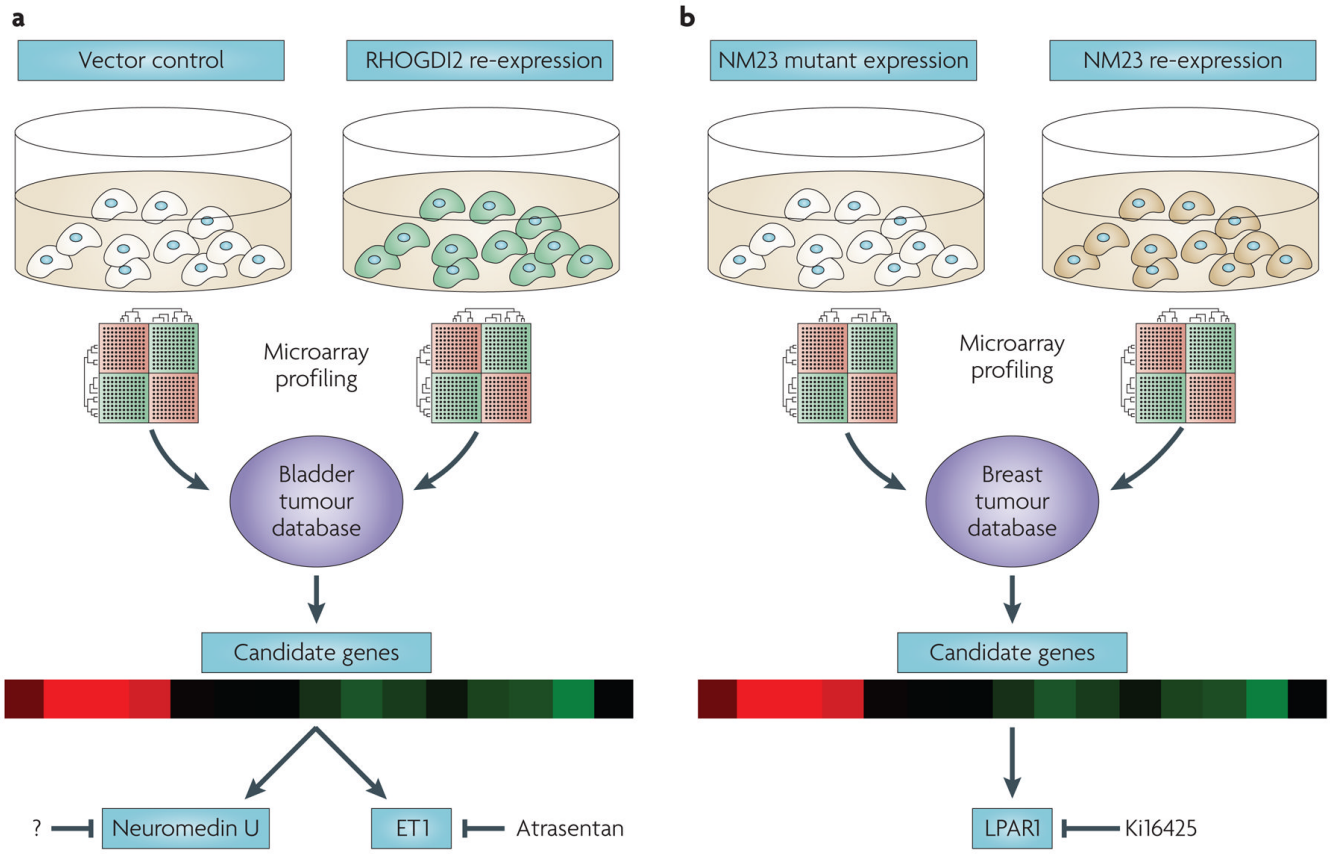


Figure 3. Targeting key genes downstream of metastasis suppressors

In the case of two metastasis suppressor genes, NM23 (REFs [40,100]) and RhoGTPase dissociation inhibitor 2 (RHOGDI2)[95,98], investigators have found by microarray expression profiling that loss of metastasis suppressor expression results in transcriptional deregulation. **a.** By re-expressing RHOGDI2 in metastatic bladder cancer cells and comparing transcripts repressed by the suppressor to genes overexpressed in invasive bladder tumours, Titus *et al.* discovered both endothelin 1 (ET1), which is druggable by an inhibitor of its receptor, atrasentan, and neuromedin U, which is a potent pro-metastatic gene that cannot yet be targeted therapeutically. **b.** By microarray profiling breast cancer cells re-expressing NM23 and mutants lacking suppressor activity, then screening differentially expressed transcripts for appropriate correlations to NM23 expression levels in human tumours[40], Horak *et al.* uncovered LPAR1, a lysophosphatidic acid receptor, among other genes, as a key downstream target potentiating motility and metastasis[100] in breast cancer cells. An antagonist of LPAR1, Ki16425, has been reported and is being evaluated for therapeutic activity[32].

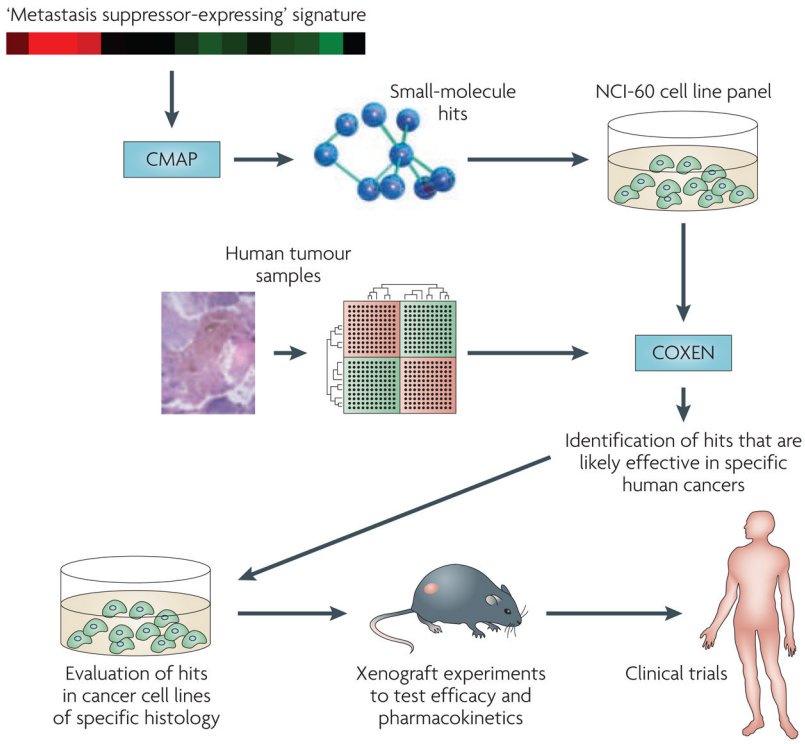
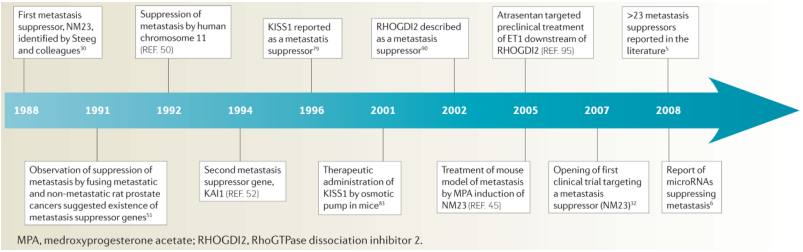


Figure 4. Targeting metastasis suppressor signatures through the Connectivity Map and COXen (Coexpression extrapolation)

Advanced informatic technologies including the Connectivity Map[103] allow for screening an entire 'metastasis suppressor-expressing cell'-type gene expression signature against gene expression changes induced by compounds, potentially systematically identifying molecules capable of inducing non-metastatic gene expression and phenotype. The list of hits is then further filtered using the COXEN algorithm, which provides a list of those suppressor signature-inducing agents that additionally have therapeutic efficacy in the NCI-60 cell line system[99] and, most importantly, predicts function in patient tumours[104]. Together, this integrated approach could deliver therapeutics based on metastasis suppressor biology to the clinic with a high likelihood of efficacy.



Timeline.
Key advances in the metastasis suppressor field

Table 1

Metastasis suppressor genes: functions and reported targeting strategies

Symbol	Alias(es)	Function(s)	Potential targeting strategy
BMP4	BMP2B	Soluble cytokine	Direct therapeutic administration of suppressor protein*
BRMS1	None	Chromatin and transcriptional regulation; regulation of gap junctions	None published at present
CTGF	CCN2, IGFBP8	Soluble cytokine	None published at present
DLC1	ARHGAP7	Regulation of RhoGTPase signalling	Re-induction of endogenous gene through HDAC inhibition[69]
KAI1	CD82, kangai 1	Inhibition of EGFR signaling; induction of senescence through interaction with DARC	Therapeutic re-induction of endogenous gene by plant extracts[60]; viral[62] and non-viral[61] gene therapy
KISS1	KiSS-1, metastin	Soluble ligand for G-protein-coupled receptor	Direct therapeutic administration of suppressor protein[83]; possibly small molecule mimetics[84]
MKK4	MAP2K4	Signal transduction	Antibody-mediated activation pathway upstream of MKK4 (REF. [122])
NDRG1	CAP43, DRG1, RTP	Unknown	Induced by iron chelators[123], p53 (REF. [124]) and PTEN expression[125]
NM23	NME1, NM23-H1	Histidine kinase activity to KSR1, decreasing Ras signalling; regulation of downstream gene expression	Re-induction of endogenous gene[42,47,48]; viral gene therapy[49]; inhibition of downstream genes[40]
RHOGDI2	ARHGDIB, LyGDI, GDID4	Regulation of Rho family member activation; regulation of downstream gene expression	Inhibition of downstream genes[95]
RKIP	PEBP1	Binds to and inhibits Raf kinase activity and downstream signalling	Epigenetic re-induction of endogenous gene[67]

* R. Anderson, unpublished data, also presented at the 2008 Meeting of the American Association for Cancer Research-Metastasis Research Society, Vancouver, Canada. *BMP4*, bone morphogenetic protein 4; *BRMS1*, breast cancer metastasis suppressor 1; DARC, Duffy chemokine receptor; EGFR, epidermal growth factor receptor; HDAC, histone deacetylase; *MKK4*, MAPK kinase 4; *RHOGDI2*, RhoGTPase dissociation inhibitor 2; *RKIP*, Raf kinase inhibitory protein.