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MINIREVIEW

Translational approaches using metastasis suppressor genes

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Abstract Cancer metastasis is a significant contributor to breast cancer patient morbidity and mortality. In order to develop new anti-metastatic therapies, we need to understand the biological and biochemical mechanisms of metastasis. Toward these efforts, we and others have studied metastasis suppressor genes, which halt metastasis in vivo without affecting primary tumor growth. The first metastasis suppressor gene identified was *nm23*, also known as NDP kinase. Nm23 represents the most widely validated metastasis suppressor gene, based on transfection and knock-out mouse strategies. The biochemical mechanism of metastasis suppression via Nm23 is unknown and likely complex. Two potential mechanisms include binding proteins and a histidine kinase activity. Elevation of Nm23 expression in micrometastatic tumor cells may constitute a translational strategy for the limitation of metastatic colonization in high risk cancer patients. To date, medroxyprogesterone acetate (MPA) has been identified as a candidate compound for clinical testing.

Keywords Nm23 · RhoGDI2 · Metastasis · Metastasis suppressor · Breast · Cancer · Histidine protein kinase

Tumor metastasis

Tumor metastasis, the movement of tumor cells from a primary tumor to colonize a distant site, is a major contributor to cancer patient morbidity and mortality. The metastatic process is complex (Chambers et al., 2002; Steeg, 2000; Welch et al., 2000). Tumor cells must invade the primary tumor border, which is thought to involve reversible changes in adhesion, protease production and motility. Tumor cells must intravasate the circulatory system, survive in the harsh environment of the bloodstream, arrest and extravasate. Sites of metastasis are determined by both physical constraints, i.e., trapping in the first capillary bed, and interactions with the vasculature. Once at a distant organ, tumor cells may die, remain dormant, or form progressively growing metastases (colonization).

The process of metastatic colonization involves both growth at a distant site, as well as angiogenesis, the formation of a vascular supply from preformed vessels. The new vasculature is often tortuous in its architecture, leaky, and exhibits interstitial pressure imbalances. From a theoretical standpoint, it has been difficult to envision "growth" at a secondary site as being distinct from that at the primary site, particularly with reference to the tumor cell. However, tumor cells encounter distinct microenvironments in the primary and secondary sites, and these interactions can facilitate or repress growth. Examples include locally produced chemokines and cytokines, and cell:cell interactions in specialized environments such as the bone, liver, lung and brain.

Metastasis is quantified by in vivo assays. Two types of xenograft assays exist, spontaneous and experimental (rev. in (Welch, 1997)). In spontaneous assays, tumor cells are injected into a site, a primary tumor forms and metastases seed out. It is preferable to inject tumor cells into an orthotopic location, the tissue of origin. The assay measures the complete process but suffers from poor quantification and requires long times post-injection for completion. In experimental metastasis assays tumor cells are injected into the bloodstream of rodents; metastases form quickly and in

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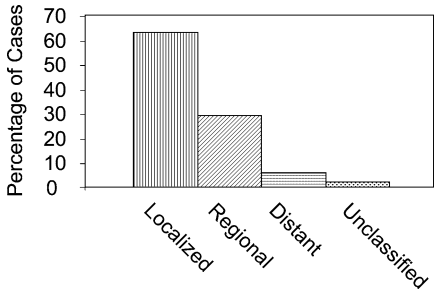

Fig. 1 Surveillance Epidemiology and End Result (SEER) program stage distribution of cancer at diagnosis. The percentage of patients at diagnosis with localized, regional or metastatic breast cancer. Data represent results of 9 SEER registries for the years 1988–2001, including all races and ages (SEER, 2005)

greater numbers, facilitating statistical analysis. In vitro assays are used to measure specific aspects of metastasis such as motility, invasion, colonization, angiogenesis etc.

One important translational question is whether the metastatic process has already been completed in cancer patients or, if not, what parts of it remain open for therapeutic development. Data collected by the Surveillance, Epidemiology and End Result (SEER) program, NCI, for breast cancer between the years of 1988–2001 are presented on Fig. 1 as an example. At the time of diagnosis and surgery patients were staged into three broad categories: those with localized disease (no evidence of progression), those with regional disease (lymph node metastases) and those with distant metastases. The proportion of US breast cancer patients in each category for the years 1988–2001 are graphed. Approximately 6% of patients had detectable distant metastases at diagnosis and surgery. This fact argues that the interruption of the metastatic process could be useful in the vast majority of patients.

Is the entire metastatic process open to intervention? Here, the regional disease data is informative. Another 29% of patients had lymph node metastases. In these patients invasion has already happened. What is unclear, because we lack imaging sensitive enough to detect only a few tumor cells, is whether intravasation and extravasation of the circulatory system and colonization to form an occult micrometastasis has occurred. These data argue that the last steps in the metastatic process, angiogenesis and the final phases of colonization, represent priority steps for therapeutic intervention.

Metastasis suppressor genes

Tumor metastasis is actually a balance of stimulatory and inhibitory regulatory signaling circuits. A number of genes inhibit both primary tumor formation and metastasis in experimental models. The metastasis suppressor genes (MSGs) form a distinct class. Many of the MSGs were identified on the basis of their reduced expression in highly metastatic tumors or cell lines, as compared to tumorigenic but poorly metastatic specimens. This stands in contrast to tumor suppressor genes, often identified by a comparison of tumor to normal tissue. Other MSGs were cloned when chromosomes or portions of chromosomes were transfected into cell lines and a dimunition of metastatic potential observed. All of the MSGs were validated by re-expression, at physiologic levels, in a metastatic cell line, which showed a reduction in metastatic potential with no significant effect on tumorigenicity as compared to controls.

Currently, thirteen MSGs have been confirmed (Table 1). Many of the MSGs have been validated in multiple cancer histologies. In no case was metastasis 100% inhibited, indicating the complexity of this process. In several cases, mice were injected with control or MSG transfected cells and the lungs harvested several days later. Tumor cells resided in the lungs several days post-injection in both groups. However, several weeks later, large metastases had formed in mice injected with control transfectants while the MSG transfectants had few metastases. This type of experiment demonstrated that MSGs may regulate metastatic colonization, the phase of tumor metastasis thought to be "open" for therapeutic intervention (Goldberg et al., 1999). Several of the MSG products are thought to function in invasion, for instance RhoGDI2. Other MSG products have novel functions, and are providing insight into metastasis. Examples include:

1. The Brms1 MSG regulates the expression of gap junction proteins and gap junctional communication, permitting low molecular weight signals to pass between adjacent cells. It can be hypothesized that this form of communication may limit metastatic colonization.
2. MSGs Nm23 and SSeCKS bind scaffold proteins, which assemble signal transduction pathway components physically and regulate the amplitude and duration of responses.
3. The MKK4 MSG product lies on the Jnk signal transduction pathway above Jnk and p38. The Jnk pathway controls apoptotic responses to stresses. It can be hypothesized that tumor cells, arriving in a distant location, may experience stresses from an altered microenvironment, lack of angiogenic connections, etc. These stresses may result in Jnk activation and apoptosis. Loss of MKK4 expression in highly metastatic tumor cells, however, may abrogate this option, promoting tumor cell survival in a foreign location (Robinson et al., 2002).

The novel functions of MSG products, as well as their regulation of colonization, part of the therapeutic window for the development of anti-metastatic strategies, suggest the hypothesis that the MSG represent interesting translational targets.

Table 1 Metastasis suppressor genes

Gene:	Histology	Known functions	References
Nm23	Breast Melanoma	See Table 3	
Kai-1	Prostate ca.	Tetraspanin	
	Breast ca.	Attenuation of EGFR Signaling	(Dong et al., 1995; Odintsova et al., 2000; Yang et al., 2001; Zhang et al., 2003)
Kiss-1	Melanoma	Precursor of peptide ligand	
	Breast ca.	Endocrine effects	(Kotani et al., 2001; Lee et al., 1996; Lee and Welch, 1997; Muir et al., 2001; Ohtaki et al., 2001; Seminara et al., 2003)
TXNIP	Melanoma	Thioredoxin binding protein	
		Vitamin D upregulated protein	(Goldberg et al., 2003)
CRSP3	Melanoma	Transcriptional co-activation	(Goldberg et al., 2003)
MKK4	Prostate ca.	Jnk pathway signaling	
	Ovarian ca.		(VanderGriend et al., in press; Yamada et al., 2002; Yoshida et al., 1999)
MKK7	Prostate ca.	Jnk pathway signaling	(VanderGriend et al. in press)
BRMS1	Breast ca.	Gap junctional communication Phosphoinositide signaling NF-kB activity	
		HDAC complex component	(Cicek et al., 2005; DeWald et al., 2005; Meehan et al., 2003; Saunders et al., 2001; Seraj et al., 2000)
SSeCKS	Prostate ca.	Scaffold for protein kinases C and A	(Xia et al., 2001)
RhoGDI2	Bladder ca.	Rho signaling?	(Gildea et al., 2002; Titus et al., 2005)
Drg-1	Colon ca.	Phosphoprotein, function unknown	(Bandyopadhyay et al., 2003)
CTGF	Colon ca.	Secreted growth factor	(Lin et al., 2005)
RKIP	Prostate ca.	Raf signaling inhibitor Phosphoethanolamine binding protein Sensitization to chemotherapy induced apoptosis	(Chatterjee et al., 2004; Keller et al., 2005)

Nm23 Metastasis suppressor gene (MSG)

The murine *nm23* cDNA was discovered using differential colony hybridization between murine K-1735 melanoma cell lines that varied in metastatic potential in vivo (Steeg et al., 1988). The *nm23* mRNA levels of two low metastatic potential cell lines were quantitatively higher than that of five related but highly metastatic cell lines. Protein levels exhibited a similar pattern (Rosengard et al., 1989). Nm23 mRNA and protein levels have been examined in many model systems, and reduced expression observed in highly metastatic specimens of a subset of these models. Nine members of the human *nm23* family (*nme*) have been reported and are found in multiple subcellular compartments.

Nm23 expression levels have been widely reported in human tumor cohorts (rev. in (Hartsough and Steeg, 2000)). Reduced Nm23 expression correlated with an aspect of high metastatic potential (i.e., reduced patient disease free or overall survival, the presence of lymph node metastases, poor tumor differentiation grade) in a subset of breast, gastric, ovarian, cervical, hepatocellular carcinomas and melanoma cohorts. In some studies no trend was evident, although the specificity of anti-Nm23 antibodies and differing scoring systems may be confounding factors. Increased soluble Nm23 expression has been reported in leukemias and lymphomas, which may result from increased erythrocyte lysis. Taken together, these studies suggest that Nm23 expression is not an independent predictive or prognostic factor for most cancer histologies. However, the studies do support the biological hypothesis that decreased Nm23 expression is related to tumor aggressiveness, which has been tested in multiple transfection experiments.

Table 2 lists studies in which transfection of *nm23-H1* or *–H2* cDNAs (or their murine homologs –M1 or M2) into a metastatically competent cell line reduced the in vivo metastatic potential of these cell lines. No significant effect on primary tumor formation was noted in the spontaneous metastasis experiments. Nm23 represents the most extensively confirmed of the MSGs. These data were recently extended by the characterization of a *nm23-M1* knockout mouse. When induced to form hepatocellular carcinoma, primary tumor size of the knockout mice did not change significantly, but the incidence of metastases increased (Boissan

Table 2 Effect of *nm23* transfection on metastasis in vivo

Cell type	Cell line	Primary tumor size	Decrease in Metsa (5)	Reference
Breast:				
	Human MDA-MB-435	same	50–90	(Leone et al., 1993)
	HumanMDA-MB-435	same	90–100	(Bhujwalla et al., 1999)
	Human MDA-MB-231	same	44–46	(Russell et al., 1998)
	Rat MTLn3	↑13%	48	(Fukuda et al., 1996)
Melanoma:				
	Murine K-1735-TK	same	52–96	(Leone et al., 1991)
	Murine B16-FE7	same	83	(Baba et al., 1995)
	Murine B16-F10	N/A	93	(Parhar et al., 1995)
	Murine MelJuSo	same	40–80	(Miele et al., 1997)
Colon:				
	Rat Colon 26	N/A	94	(Tagashira et al., 1998)
	HT-29	N/A	89	(Suzuki et al., 2004)
	Oral Squamous:			
	Human LMF4	N/A	73–98	(Miyazaki et al., 1999)

[a]Decrease in the incidence of metastases in vivo, as compared to mean of control transfectants. N/A, Primary tumor size data not measured.

et al., 2005). To date, metastasis data do not exist for the other homologs of nm23.

Several in vitro phenotypes have been reported for control- and *nm23-H1*/*H2* transfected isogenic tumor cell lines. Signal responsiveness to TGF-β in colonization (Leone et al., 1993), or to IGF, serum, PDGF, LPA, etc. in motility, was diminished in the *nm23* transfectants (Kantor et al., 1993). A three-dimensional culture system in extracellular matrix (Matrigel) was used to study differentiation. Nm23-H1 transfectants, but not control transfectants exhibited morphological (ascinus formation) and biochemical (synthesis and basolateral secretion of basement membrane proteins, synthesis of sialomucin) aspects of breast differentiation (Howlett et al., 1994). For the nm23-DR homolog, in vivo metastasis data have not been reported. Rather, overexpression of Nm23-DR alters neuroblastoma differentiation and sensitivty to apoptosis induction (Amendola et al., 1997; Negroni et al., 2000). Two themes have emerged in these data, the role of Nm23 in differentiation and its role in limiting or directing signal transduction. The role of Nm23 in differentiation was confirmed in *Drosophila* with its homolog *abnormal wing discs* (*awd*). Loss of expression or mutation of *awd* resulted in widespread abnormalities centered on the outgrowth of presumptive adult epithelial tissues from the imaginal discs (Biggs et al., 1988; Dearolf et al., 1988a,b; Xu et al., 1996).

Biochemical mechanism of Nm23 metastasis suppression

To date, the biochemical pathway by which elevated Nm23 expression suppresses metastasis is unknown,despite extensive study. Nm23 proteins possess multiple biochemical functions. These include:

(1) Interactions with numerous proteins, including the integrin cytoplasmic domain-associated protein 1α (ICAP-1α) (Fournier et al., 2002), Epstein-Barr virus nuclear protein EBNA-3C (Subramanian et al., 2001), the Prune protein (Pn) (D'Angelo et al., 2004), the centrosomal kinase Aurora-A/STK15 (Du and Hannon, 2002), the Lbc proto-oncogene (Iwashita et al., 2004), intermeidate filaments (Otero, 1997; Roymans et al., 2000), map kinases (Moon et al., 2003), telomeres (Nosaka et al., 1998), T4 phage proteins (Shen et al., 2004) etc. Because Nm23 is an inherently "sticky" protein, protein:protein interactions are not considered biologically relevant unless an alteration in the function of one of the binding partners is identified.
(2) A nucleoside diphosphate kinase (NDP kinase) activity, in which Nm23 removes the terminal phosphate from a NTP to autophosphorylate its own histidine 118, then transfers the phosphate to a NDP to recreate a NTP (Wallet et al., 1990);
(3) DNA- based activities, including binding to transcription factors (Cheng et al., 2002), binding DNA and altering transcription (Levit et al., 2002; Michelotti et al., 1997; Postel et al., 1993), binding single stranded DNA (Hildebrandt et al., 1995), Granzyme A-activated DNase (Fan et al., 2003), and an exonuclease function (Ma et al., 2004). Several functions in this section have been debated: Evidence for and against a direct myc transcriptional activity have been published (Michelotti et al., 1997; Postel et al., 1993). A uracil processing DNA repair function (Postel and Abramczyk,

2003) has been reported; other studies attribute this activity in its entirety to Ung contamination (Bennett et al., 2004; Kumar et al., 2004). Transcriptional modulation via participation in a G-quadruplex DNA structure has been reported and retracted (Grand et al., 2004). Many of the issues surrounding this controversy involve protein purification, and confirmation of the remaining activities is warranted.

(4) Histidine protein kinase activity (Engel et al., 1995; Freije et al., 1997; Hartsough et al., 2002; Inoue et al., 1996; Lu et al., 1996; Wagner and Vu, 1995; 2000b; Wagner et al., 1997). Histidine protein kinases are poorly understood in mammalian biology, but transfer a phosphate from a protein phospho-histidine intermediate to a substrate protein.

Given the plethora of potential biochemical activities for Nm23 proteins, a system was needed to determine which correlated with biological function. We conducted site directed mutagenesis of nm23-H1. Wild type and site directed mutant nm23-H1 constructs were transfected into MDA-MB-435 breast carcinoma cells, and in vitro motility used as a readout of one aspect of metastasis (MacDonald et al., 1996). The P96S and S120G mutations, but not the S44A mutation, impaired the motility suppressive capacity of Nm23-H1 to either autotaxin or the combination of factors in serum. Further studies correlated these mutations with effects on the proposed biochemical activities of Nm23 (Table 2). A lack of correlation of NDP kinase activity and motility suppression was observed. Although the data are incomplete, no correlation of motility suppression with DNA transactivation, DNA cleavage or Tiam GEF activity were noted. Nm23 phosphorylation of isoprenoid pyrophosphates (Wagner and Vu, 2000a) correlated with motility suppression and stands as a candidate mechanistic activity. Another biochemical activity that may be germane to motility suppression is an interaction with G-proteins, as evidenced by muscarinic channel gating (Otero et al., 1999).

Among the candidate activities correlating with Nm23 functional mutations, we focused our attention on the histidine protein kinase activity of Nm23. Histidine protein kinases are well described in prokaryotes and lower eukaryotes, where they form the "two component" signal transduction system. Several features distinguish histidine protein kinases from the more conventional serine, threonine and tyrosine kinases: (1) histidine is a high energy bond; (2) phosphohistidine is acid labile, and therefore difficult to detect in conventional gel systems; (3) histidine kinases form a phospho-histidine intermediate, which then transfers the phosphate to the substrate. Nm23 has been associated with several types of histidine protein kinase activities. Substrates such as succinyl thiokinase (STK), ATP-citrate lyase, and recombinant Nm23's involve a histidine-histidine phosphotransfer, while aldolase involves a hisitine-aspartate similar to the two-component pathways (Wagner and Vu, 2000b).

Phosphorylation of the Map kinase scaffold, the Kinase suppressor of ras (Ksr), involves a histidine-serine transfer (Hartsough et al., 2002). In other model systems, nucleoside diphosphate kinase 2 was involved in *Arabidopsis* phytochrome signaling (Choi et al., 1999); interaction of Ndk2 with phytochromes increased its kinase activity to myelin basic protein (Shen et al., 2005). Nm23-H1 has also been reported to fuse at the mRNA level with GAPDH, resulting in a longer protein with kinase activity. Among the substrates of this hybrid protein are phosphoglycerate mutase (PGM), which represents a histidine-histidine phosphotransfer (Engel et al., 2004). Nm23-H2 has been reported to bind heterotrimeric G proteins, and to phosphorylate the Gβ subunit (Cuello et al., 2003; Hippe et al., 2003).

The P96S mutant of Nm23-H1 showed impaired motility suppression, and a concurrent impairment of function in assays for histidine kinase activity (Table 3). For the S120G mutation, the relationship is more complex. Initial characterization indicated diminished autophosphorylation of Nm23, but wild type rates of phosphotransfer. Thus, if equal amounts of autophosphorylated wild type and S120G Nm23 proteins were added to histidine protein kinase assays, this mutant could function properly, as reported for the phosphorylation of STK and Ksr. Impaired histidine protein kinase activity was nevertheless observed for recombinant Nm23s and Aldolase. The S44A mutant Nm23-H1, which was motility suppressive, was active in all histidine protein kinase assays reported. These data indicate a good correlation of histidine protein kinase activity and motility suppression, suggesting the hypothesis that they are functionally related.

A posited objection to the histidine kinase activity of Nm23 contends that the size of the Nm23 histidine containing pocket, determined by X-ray crystallography, is too small to accommodate a protein. Several factors may be germane. First, the structures of Nm23 in solution may be different than in crystals. Second, work in *Drosophila* suggested that only a small percentage of normal total NDP kinase activity is needed to restore normal fly development, suggesting that a minor subpopulation of Nm23 with an altered function may be biologically potent (Xu et al., 1996). Third, two new structures of Nm23 protein have been described in experimental studies, that of a molten globule (Lascu et al., 2000) and as a hybrid with GAPDH. If these or other structures exist in nature, the problem of active site size could be solved.

Many of the substrates used in histidine protein kinase assays represent "ice bucket" biochemical pathways, and are not thought to be candidate physiological substrates for Nm23-H1 in metastasis suppression. Two proteins, however, show interesting possible connections. Nm23-H1 phosphorylated an aspartate residue of Aldolase C (Wagner and Vu, 2000b). Aldolase C is primarily found in brain tissues, but is

Table 3 Effect of site directed mutagenesis on Nm23-H1 biological function in motility, and its biochemical characteristics

Assay:	Wild type	Mutant P96S	Mutant S44A	Mutant S120G	Mutant H118F
MDA-MB-435 Motility: (MacDonald et al., 1996)					
0.5% FCS	0.4–11.4	10.5–41.9	1.0–11.2	42.2–43.4	ND
5mM ATX	0–3.8	28.2–33.1	5.4–10.1	29.4	ND
NDP Kinase Activity: (Freije et al., 1997)					
	+++	+++	+++	+++	−
Nm23-H1 Autophosphorylation: (Freije et al., 1997)					
	+++	+++	+++	++	−
Nm23-H1 Histidine protein kinase: (Freije et al., 1997) (Hartsough et al., 2002; Wagner et al., 1997)					
STK	+++	+	+++	+++	−
rNm23-H2	+++	+	+++	++	−
GST-Nm23-H1	+++	-	+++	++	−
Aldolase	+++	+	ND	+	
Ksr	+++	+	ND	+++	−
Nm23-H1 Kinase of Isoprenoid pyrophosphates: (Wagner and Vu, 2000a)					
GPP	+++	+	ND	++	ND
FPP	+++	+	ND	+	ND
Desensitization of Muscarinic K+ channel gating: (Otero et al., 1999)					
	+++	+++	+++	−	ND
Nm23-H1 C-terminus DNA Transactivation: (Cho et al., 2001)					
	+++	+++	ND	+++	−
Nm23-H2 DNA Cleavage: (Postel et al., 2002)					
	+++	ND	ND	+++	+++
Nm23-H1 inhibition of Tiam1 GEF activity (Otsuki et al., 2001)					
	+++	+++	ND	ND	+++
Nm23-H1 Binding to Prune and modulation of Prune PDE activity: (D'Angelo et al., 2004)					
	+++	+++	ND	+	ND

Note. ND, Not determined.

also present in certain tumors. The effects of aspartate phosphorylation on Aldolase C function are unknown. However, Aldolase C is reported to bind Phospholipase D2, a well known signal transduction intermediate (Kim et al., 2002).

Recently, an interaction of Nm23-H1 with Ksr was reported (Hartsough et al., 2002). Ksr, initially identified in *Drosophila* and *Caenorhabditis elegans* systems, showed inactivating mutations that suppressed the phenotypic effects of activated Ras (Kornfield et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995). These genetic studies placed Ksr either upstream or parallel to Raf in signal transduction. Biochemical studies are still investigating Ksr function. The best hypothesis to date is that Ksr serves as a scaffold protein for the MAP kinase pathway, providing docking sites, intracellular translocation, and specificity in signaling. Ksr binds Raf-1, MEK1/2, Erk1/2, 14-3-3, Hsp90, p59cdc37, heterotrimeric G proteins, c-Tak and Nm23-H1 in support of its scaffold function (Bell et al., 1999; Cacace et al., 1999; Denouel-Galy et al., 1997; Hartsough et al., 2002; Jacobs et al., 1999; Muller et al., 2001; Stewart et al., 1999; Xing et al., 1997; Yu et al., 1997). Antibodies to Nm23 co-immunoprecipitated Ksr from both 293T and human MDA-MB-435 breast carcinoma cells. Autophosphorylated recombinant Nm23-H1, when incubated with Ksr immunoprecipitated from transiently transfected 293T cells, phosphorylated full length and N-terminal Ksr, but not C-terminal Ksr or the related protein Raf-1. The Ksr phosphorylation was determined to be serine by phosphoaminoacid analysis, and HPLC analysis of tryptic Ksr peptides showed two peaks of phosphorylation. Site directed mutagenesis confirmed Nm23-H1 phosphorylation of Ksr serines 392, a 14-3-3- binding site, and 434. Serine 434 is a known site of Ksr phosphorylation in vivo, but was previously unassociated with a kinase. Importantly, control transfectants of the MDA-MB-435 cell line exhibited Erk1/2 activation, as measured by western blot using antibodies to phospho-specific and total Erk proteins. Overexpression of *nm23-H1*, in addition to inhibiting metastasis in vivo and motility in vitro, resulted in diminished Map kinase activation, although total Erk1/2 levels were comparable. The P96S kinase-deficient Nm23-H1 transfectant exhibited relatively high levels of activated Map kinase, suggesting that the histidine protein kinase activity of Nm23-H1 is needed for suppression of Map kinase activation. Taken together, we hypothesize that overexpression of Nm23-H1 results in higher histidine protein kinase activity, which phosphorylates Ksr in a novel pattern and diminishes its ability to facilitate Map kinase activation.

The consequences of Nm23-H1:Ksr on the Ksr scaffold function have been investigated (Salerno et al., 2005). In high Nm23-H1 expressing breast carcinoma cells, Ksr co-immunoprecipitated quantitatively higher levels of the Heat shock protein 90 (Hsp90) chaperone protein than in control transfectants; binding of Mek to the Ksr scaffold was modestly altered while no effect was observed on the binding of other scaffold client proteins such as 14-3-3. Hsp90 influences both the initial folding and ultimate degradation of client proteins via an assembly of co-chaperone binding proteins. In agreement with this function, degradation of Ksr was increased in high Nm23-H1 expressing cells and these cells were less sensitive to growth inhibition by the Hsp90 inhibitor 17-AAG. The data demonstrated that the Nm23-Ksr interaction can alter the client binding and scaffold function of Ksr.

Proteins that bind Nm23-H1 are of interest mechanistically. Both the Prune and the Epstein-Barr virus EBNA-3C protein physically bind Nm23-H1. In both cases, binding abrogates the motility suppressive capacity of Nm23-H1 (D'Angelo et al., 2004; Subramanian et al., 2001). Thus, metastatic potential is not as simple as Nm23 expression level and the relative levels of binding proteins may need to be considered.

Translational hypotheses for metastasis suppressor genes

Our ability to control metastatic disease has improved only incrementally in decades of clinical development and testing. Can we target the metastatic process, particularly those later events in angiogenesis and metastatic colonization, rather than use standard cytotoxic chemotherapy approaches? Two translational approaches based on MSG have been reported.

1. Restoration of MSG expression in micrometastatic tumor cells.

We postulate, based on cohort and transfection data, that overexpression of Nm23-H1 in micrometastatic breast or other tumor cells may result in differentiation, and the inhibition of colonization and invasion, with a therapeutic benefit. In other words, we propose that breast cancer patients whose tumors express low levels of Nm23 receive traditional resection, radiation therapy and chemotherapy, and then go to a maintenance therapy aimed at elevation of the Nm23 expression in remaining micrometastatic tumor cells. This type of therapy would be life long, and would require a drug that is relatively nontoxic with an easily tolerated dose and schedule.

The question to be answered is how to elevate Nm23-H1 expression in vivo. Using tumor cell lines, several agents have been reported to elevate Nm23 expression. All-trans retinoic acid was reported to elevate Nm23 expression in a hepatocellular carcinoma cell line (Liu et al., 2000); we have repeated this observation using MDA-MB-231 human breast carcinoma cells, but were unable to reproduce the effect with the clinically used fenretinide (unpublished data). Another study reported that gamma-linolenic acid (GLA) elevated the Nm23 expression of MDA-MB-231 breast carcinoma

cells (Jiang et al., 1988). Elevation of Nm23-H1 expression by the DNA methylation inhibitor 5-aza-deoxycytidine was observed in cell lines but alterations in the methylation status of CpG islands in the nm23-H1 promoter was not observed in tumors (Hartsough et al., 2001), suggesting that this observation was an in vitro artifact. Estrogen was reported to elevate Nm23 expression of several breast carcinoma cell lines; we note that many metastatic breast tumors are ER- additionally we failed to repeat the published trend in MCF-7 breast carcinoma cells using physiologic doses of estrogen.

We used restriction mapping of the *nm23-H1* promoter in breast cell lines to map a ∼400 bp promoter fragment which contributed to high- versus low *nm23* expression levels (Ouatas et al., 2002). This fragment contained a cassette of transcription factors found in the MMTV-LTR, and the WAP and milk protein promoters. Site directed mutagenesis of the sites confirmed their participation in the regulation of reporter gene expression driven by the *nm23-H1* promoter (Ouatas et al., 2002). Based on the MMTV-LTR data, where the cassette of transcription factors are regulated by steroids, we investigated the effect of steroids on Nm23-H1 expression. Dexamethasone elevated the Nm23-H1 expression of two metastatic breast carcinoma cell lines, MDA-MB-231 and MDA-MB-435, only when cultured in charcoal stripped serum containing medium, devoid of endogenous steroids (Ouatas et al., 2003). These data offered a proof of concept that glucocorticoids could elevate Nm23-H1 expression, but indicated that dexamethasone was effective at physiologic, but not pharmacologic levels.

Medroxyprogesterone acetate (MPA) is a progestin used at low concentrations in both birth control and hormone replacement therapy. At high concentrations it has been used clinically for advanced breast and endometrial cancers. MPA binds to receptors for progesterone (PR), androgen (AR) and glucocorticoids (GR), the latter via both a traditional DNA binding and a nontraditional DNA-independent mechanism. MPA was tested using MDA-MB-231 and −435 breast carcinoma cells, which are estrogen and progesterone receptor negative. At doses of 50–200 nM, MPA elevated Nm23-H1 expression and inhibited soft agar colonization (Ouatas et al., 2003). Elevation of Nm23-H1 expression was inhibitable by the PR/GR antagonist RU-486, and was not observed using an androgen, indicating that MPA was utilizing the glucocorticoid receptor.

The effect of MPA on the elevation of Nm23-H1 expression was tested in an in vivo model system for the outgrowth of micrometastases (Palmieri et al., 2005). MDA-MB-231 breast carcinoma cells were injected into nude mice via the tail vein and housed for one month. At this time, the lungs of several sacrificed mice were examined histologically and found to have micrometastases as scored by a pathologist. The remaining mice were randomized to vehicle control or doses of MPA, given in a one month induction dose and then a monthly or bimonthly maintenance dose. Metastases developed in 100% of control mice versus 64–73% of MPA treated mice. The mean number of gross metastases per mouse was reduced by 33–62% depending on the MPA dose. Large metastases (> 3mm in any diameter) were reduced by 55–75% in the MPA treated mice. Thus, MPA inhibited the incidence, number and size of metastases in this model.

To ascertain if MPA elevated Nm23 expression in vivo, mouse lungs were sectioned and stained for Nm23 immunohistochemically. Staining was evaluated relative to the lung parenchyma. Nm23 expression levels higher than the adjacent parenchyma were observed in 13% of metastases formed in the control arm, but 41–43% of the 1–2 mg MPA doses (Palmieri et al., 2005). The data suggest that elevation of Nm23 expression in vivo occurred and contributed to the dimunition of metastasis.

For any potential therapy, the evaluation of potential side effects is of paramount importance. MPA is known to have multiple functions, some of which are stimulatory and therefore of concern. Mice were evaluated for mammary fat pad histology, weight gain, fat versus lean body mass, and bone density. Of these parameters, weight gain was the only significantside effect observed in the MPA arms (Palmieri et al., 2005). Collectively, the data identify MPA as a GR agonist for the elevation of Nm23-H1 expression and limitation of metastatic colonization. Clinical testing of MPA for triple-negative (ER-, PR-, Her-2 negative) breast cancer is under consideration.

2. Identification of "drug-able" gene expression alterations in low MSG expressing tumor cells.

Low MSG expressing tumor cells are at the highest risk for progression. The Theodoresceu laboratory, using microarray technology, identified 40 genes overexpressed in control- as compared to the RhoGDI2 MSG transfected bladder carcinoma cells. The expression of the 40 genes was then determined in a cohort of 20 bladder carcinomas, using tumor stage as an indicator of aggressiveness. This filter was applied to ensure that genes derived from cell line experiments were germane to actual cancers. Of these genes, endothelin (ET-1) was coordinately overexpressed in the control bladder line and high grade tumors (Titus et al., 2005). The endothelin axis is composed of several members that can be expressed by both cancer and normal cells resulting in both autocrine and paracrine interactions favoring growth and angiogenesis. An inhibitor of the endothelin-a receptor, atrasentan, was tested for efficacy in low RhoGDI expressing tumor cells. No effect of atrasentan was reported on tumor cell growth in vitro or primary tumor formation in vivo. Treatment of mice bearing control bladder carcinoma cells with atrasentan altered the incidence of lung metastases from 53% in

vehicle controls to 5% in the atrasentan arm (Titus et al., 2005). Both the mean number of metastases per mouse and size of metastases were also reduced by atrasentan. Thus, a gene expression alteration common in low RhoGDI2 MSG expressing cells was targeted, rather than the actual MSG itself. Similar studies with other MSGs may yield additional appealing molecular targets.

Conclusion

Nm23 represents the most widely validated metastasis suppressor gene, based on transfection and knock-out mouse strategies. The biochemical mechanism of metastasis suppression is unknown and likely complex. Two leads of intense interest are binding proteins and a histidine kinase activity. Elevation of Nm23 expression in micrometastatic tumor cells may constitute a translational strategy for the limitation of metastatic colonization in high risk cancer patients. To date, medroxyprogesterone acetate (MPA) has been identified as a candidate compound for clinical testing.

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