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Basic and Translational Advances in Cancer Metastasis: Nm23

Taoufik Ouatas,[1,2] Massimiliano Salerno,[1] Diane Palmieri,[1] and Patricia S. Steeg[1]

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Cancer metastasis is a significant contributor to breast cancer patient morbidity and mortality. 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 confirmed was *nm23*, also known as NDP kinase. Using in vitro assays, *nm23* overexpression resulted in reduced anchorage-independent colonization in response to TGF-β, reduced invasion and motility in response to multiple factors, and increased differentiation. We hypothesize that the mechanism of action of Nm23 in metastasis suppression involves diminished signal transduction, downstream of a particular receptor. We hypothesize that a histidine protein kinase activity of Nm23 underlies its suppression of metastasis, and identify candidate substrates. This review also discusses therapeutic options on the basis of reexpression of metastasis suppressors.

KEY WORDS: Nm23; metastasis; suppressor; breast.

BREAST CANCER METASTASIS

Breast cancer is the leading cancer diagnosed in American women, and the second largest cause of their cancer deaths, second only to lung cancer. Its progression is indicative of many other cancer cell types. The basic steps of breast cancer progression are diagrammed in Fig. 1. The normal breast consists of a ductal "tree" ending in lobules, where milk is produced. In cross-section the ducts and lobules contain two concentric layers of cells. The inner layer of cells, the luminal epithelia, produce milk and are thought to be the source of most breast cancers. Luminal cells are surrounded by a layer of breast myoepithelial cells which, in turn, is surrounded by a basement membrane. Small gaps between myoepithelial cells enable luminal epithelial cells to contact the basement membrane as well. The breast changes in response to hormonal signals throughout life, including puberty, pregnancy, lactation, involution, and menopause. Histologic changes thought to represent premalignancy are found on biopsies conducted for suspicious mammograms. Typical hyperplasia, additional normal appearing cells in cross-section, confer a low risk for developing invasive cancer. Atypical ductal hyperplasia involves extra abnormal appearing cells which do not meet the full criteria for a diagnosis of cancer, but confer an approximate fourfold risk for the development of invasive cancer. Ductal carcinoma in situ (DCIS) consists of cancer cells growing within the basement membrane. Detection of DCIS has skyrocketed because of mammographic screening. Treatment options vary to include lumpectomy/radiation or mastectomy, with additional hormonal therapy, and overall mortality is low, 1–3% in most studies. A diagnosis of infiltrating ductal or invasive carcinoma is made when cancer cells invade the basement membrane. In this case, invasive breast cancer is categorized by whether evidence of metastasis to the regional lymph nodes is apparent, either by a lymph node dissection or by a sentinal node examination. Node-negative cancer carries a fairly good prognosis, with 70% of patients experiencing no further relapse. These patients are subjected to chemo-, radiation, and/or hormonal therapy. The situation changes dramatically for the worse in lymph-node-positive disease, where 70% of patients will recur. Only about 6% of patients have detectable distant metastases at the time of diagnosis and surgery. For these patients, as well as the 30% of node-negative and 70% of node-positive patients

[1] Women's Cancers Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland.

[2] To whom correspondence shoud be addressed at Building 10, Room 2A33, NIH, Bethesda, Maryland 20892; e-mail: taoufik@mail.nih.gov.

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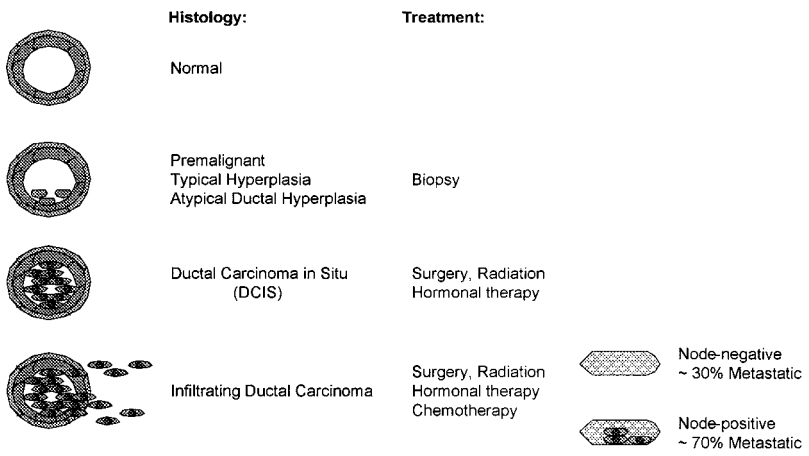

Fig. 1. Schematic of breast cancer development. The normal duct/lobule, on cross-section, consists an inner layer of luminal epithelial cells, surrounded by myoepithelial cells and a basement membrane. Progression through premalignancy, ductal carcinoma in situ to invasive cancer is thought to require multiple mutations and long time spans. It is with metastasis that patient prognosis becomes poor.

who develop metastatic disease, treatment regimens are generally ineffective. While combination chemotherapy has shown clinical responses and improved disease-free survival, overall survival has not improved sufficiently. The first molecularly targeted drug to increase overall survival in the metastatic setting is Herceptin (Slamon *et al*., 2001). One clear approach to limiting the devastation of cancer is to identify it before metastasis occurs. The other approach is to understand the metastatic process and develop effective strategies to halt it.

The metastatic process is complex (Chambers *et al*., 2002; Steeg, in press; Welch *et al*., in press). Cells must invade the primary tumor border, which is thought to involve reversible changes in adhesion, protease production, and motility. Cells must intravasate the circulatory system, survive in the harsh environment of the bloodstream, arrest, and extravasate. Both trapping in the first capillary bed encountered and site-specific metastasis occur in breast and other cancers. Once in a distant organ, cells can form a small prevascular lesion in which diffusion supplies nutrients. Tumor cells also exhibit dormancy, in breast cancer for up to 30 years, and the molecular causes are unknown. Recently, in vivo videomicroscopy was used to identify dormant cells (Naumov *et al*., 2002). These cells, when harvested, were capable of regrowth and tumor formation. Tumor outgrowth to form detectable metastases capable of compromising organ function involves angiogenesis and colonization. Metastatic colonization is a poorly described function, but may involve aberrant colonization responses to cytokines, chemokines, and other signals. A hallmark of metastasis is the redundancy of mechanistic alternatives. For instance, numerous proteases can facilitate cell movement across the basement membrane, and a plethora of angiogenesis-stimulating factors exist. One approach to this complexity is to identify common signaling or regulatory events, and inhibit them.

Metastasis suppressor genes have contributed to our understanding of the metastatic process, and represent valuable therapeutic targets. These genes are often identified by their reduced expression in a metastatic tumor cell line, as compared to a related, less metastatic tumor cell line. Proof of metastasis suppressor activity was shown by transfection experiments in which the gene is reexpressed in a low-expressing, metastatically competent tumor cell line. Upon in vivo injection, the transfectants exhibit comparable primary tumor incidence and size to control transfectants, but develop significantly less metastases. To date, seven metastasis suppressor genes have been confirmed—*nm23*, *Kiss1*, *Kai1*, *Brms1*, *E-cadherin*, *Maspin*, and *MKK4* (Yoshida *et al*., 2000). While two of these genes have known functions in halting invasion, the others were unexpected and have revealed new facets of the regulation of the metastatic process. This review concentrates on data supporting the *nm23*/NDP kinase gene as a metastasis suppressor.

Nm23 DISCOVERY AND TUMOR EXPRESSION PATTERNS

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. Eight members of the human *nm23* family (*nme*) have been reported and are found in multiple subcellular compartments (Lacombe *et al*., 2000).

Nm23 expression levels have been widely reported in human tumor cohorts (reviewed in Hartsough and Steeg, 2000). Reduced Nm23 expression correlated with an aspect of high metastatic potential (i.e., reduced disease-free or overall survival, presence of lymph node metastases, poor differentiation grade) in a subset of breast, gastric, ovarian, cervical, hepatocellular carcinomas and melanoma cohorts. In other cancer types, and in a subset cohort studies generally, either no trend or the opposite trend was reported.

Several solid tumor cohort studies are of interest: In breast cancer a large study reported Nm23-H1 expression in lymph-node-negative cancers. Low Nm23-H1 expression correlated with poor patient survival in this setting, as did other indicators of metastatic potential (high microvessel density, for example) (Heimann *et al*., 1998; Heimann and Hellman, 2000). Several reports indicate correlations of Nm23 expression and sensitivity to chemotherapy, suggesting that Nm23 can not only predict the natural history of cancer, but selected drug responses as well (Iizuka *et al*., 1999; Scambia *et al*., 1996; Wang *et al*., 2000). Mechanistic analyses of this trend, while preliminary, suggest a functional role for Nm23 (Ferguson *et al*., 1996; Iizuka *et al*., 2000). In neuroblastoma, where increased Nm23 expression correlated with aggressive disease, *nm23* mutations were reported (Chang *et al*., 1994; Leone *et al*., 1993).

Taken together, these studies suggest that Nm23 expression is not an independent predictive or prognostic factor for breast cancer. However, the studies do support the biological hypothesis that decreased Nm23 expression is related to tumor aggressiveness, which was tested in transfection experiments.

METASTASIS SUPPRESSOR FUNCTION

The hypothesis that Nm23 possesses metastasis suppressive activity was tested in transfection experiments. Table I lists those studies in which transfection of *nm23* cDNA into a metastatically competent cell line reduced its in vivo metastatic potential. Where studied, primary tumor size was not coordinately reduced, indicating a metastasis and not a tumor suppressor function. In no study did *nm23* transfection completely abrogate metastasis, supporting the heterogeneity and complexity of this process. However, the number of positive transfection studies, and the diversity of cancer cell types used strongly support a metastasis suppressive function for *nm23*. It is not known if all *nm23* family members are metastasis suppressive, or equally so.

Analysis of the in vitro phenotypes exhibited by control- and *nm23*-transfectants points to signal transduction as a common theme. Signal responsiveness to TGF-β in colonization, or to IGF, serum, PDGF, LPA, etc., in motility, is diminished in the *nm23* transfectants. Signaling in response to basement membrane proteins was altered in an in vitro model of differentiation. This model used three-dimensional culture of breast cells in basement membrane proteins, which permitted morphological (ascinus formation) and biochemical (synthesis and basolateral secretion of basement membrane proteins, synthesis and luminal secretion of sialomucin) aspects of breast differentiation. *nm23-H1* transfectants, but not control transfectants or the parental cell line, exhibited all the listed aspects of differentiation with the exception of luminal sialomucin secretion (Howlett *et al*., 1994). Other studies have investigated a role for Nm23 in differentiation in *Drosophila* development and neuronal cell types, and this subject is reviewed elsewhere in this volume.

BIOCHEMICAL FUNCTION(S) UNDERLYING 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) their interaction with numerous proteins; (2) a nucleoside diphosphate kinase (NDP kinase) activity, in which Nm23 removes the terminal phosphate from an NTP to autophosphorylate its own histidine 118, then transfers the phosphate to an NDP to recreate an NTP (Wallet *et al*., 1990); (3) DNA transactivation (Hildebrandt *et al*., 1995; Ma *et al*., 2002; Michelotti *et al*., 1997; Postel *et al*., 1993); (4) DNA nuclease (Ma *et al*., 2002; Postel, 1999); (5) serine or histidine protein kinase (Engel *et al*., 1995; Freije *et al*., 1997; Hartsough *et al*., in press; Inoue *et al*., 1996; Lu *et al*., 1996; Wagner *et al*., 1997; Wagner and Vu, 1995, 2000a,b). These activities are reviewed extensively elsewhere in this volume.

Given the plethora of potential biochemical activities for Nm23 proteins, a system was needed to determine

Table I. Effect of nm23 Transfection on Metastasis In Vivo

Cell type	Cell line	Primary tumor size	Decrease in Mets[a]	Refs.
Breast				
	Human MDA-MB-435	same	50–90%	Leone *et al*. (1993)
	Human MDA-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	na	93%	Parhar *et al*. (1995)
	Murine Mel JuSo	same	40–80%	Miele *et al*. (1997)
Colon				
	Rat Colon 26	na	94%	Tagashira *et al*. (1998)
Oral Squamous				
	Human LMF4	na	73–98%	Miyazaki *et al*. (1999)

Note. na: primary tumor size data are not measured.
[a] Decrease in the incidence of metastases in vivo, as compared to mean of control transfectants.

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 an in vitro readout of one aspect of metastasis (MacDonald *et al*., 1996). The *P96S* and *S120G* mutations, but not the *S44A* mutation, impaired the motility suppressive capcity of Nm23-H1 to either autotaxin or the combination of factors in serum. Numerous reports have then determined whether these mutations affect proposed biochemical functions of Nm23 (Table II). A lack of correlation of NDP kinase activity and motility suppression was observed. Although the data are incomplete, no correlation of motility suppression and DNA transactivation, DNA cleavage, or Tiam GEF activity were noted. Nm23 phosphorylation of isoprenoid pyrophosphates (Wagner and Vu, 2000ab) 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, we focused our attention on the histidine protein kinase activity of Nm23, as it appeared to correlate with motility suppression. 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 phosphohistidine intermediate, which then transfers the phosphate to the substrate. For the *P96S* mutation of *nm23-H1*, which showed impaired motility suppression, all assays for histidine protein kinase activity showed a concurrent impairment of function (Table II). This includes an Nm23-H1 phosphohistidine transfer to the histidine of succinyl thiokinase (STK) and recombinant Nm23s, to the aspartate of aldolase, and to a serine of kinase suppressor of Ras (Ksr) (Table II). The *S120G* mutation is more complex, as our initial characterization indicated a deficit in the autophosphorylation portion of the pathway. 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.

One objection to this activity has been 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)

Table II. 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*., in press; Wagner *et al*., 1997)					
STK	+++	+	+++	+++	−
rNm23-H2	+++	+	+++	++	−
GST-Nm23-H1	+++	−	+++	++	−
Aldolase	+++	+	ND	+	
Ksr	+++	+	ND	+++	−
Nm23-H1 kinase of isoprenoid pyrophosphates (Wagner and Vu, 2000a,b)					
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	+++

Note. ND: not determined.

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, 2000a,b). Aldolase C is primarily found in brain tissues, but is 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).

We recently characterized the interaction of Nm23-H1 with Ksr (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*., in press; Jacobs *et al*., 1999; Muller *et al*., 2001; Stewart *et al*., 1999; Xing *et al*., 1997; Yu *et al*., 1997). Antibodies to Nm23 coimmunoprecipitated 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 392, a 14-3-3-binding site, and Ksr serine 434 in conjunction with the serine 392 mutation. Serine 434 is a known site of Ksr phosphorylation in vivo, but was previously unassociated with a kinase. Current experiments are determining the effect of phosphorylation at these sites on Ksr scaffold function,

intracellular movement, and Map kinase activation. 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.

CANDIDATE AGENTS FOR ELEVATION OF Nm23-H1 EXPRESSION

We postulate, on the basis of 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 patients with breast cancer whose tumors express low levels of Nm23 go through surgery and conventional therapy, and then go to a maintenance therapy aimed at elevation of the Nm23 expression in remaining micrometastatic tumor cells. This type of therapy is envisioned for life, and would require a drug that is relatively nontoxic and 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 γ-linolenic acid (GLA) elevated the Nm23 expression of MDA-MB-231 breast carcinoma cells (Jiang *et al*., 1988).

We reported that the DNA methylation inhibitor 5-aza-deoxycytidine elevated Nm23-H1 expression in metastatically competent human breast carcinoma cell lines (Hartsough *et al*., 2001). Elevation of Nm23 expression in these lines was accompanied by reduced DNA methylation in a CpG island of the *nm23-H1* promoter; however, in a study of human breast tumors, altered methylation of this CpG island was infrequent. These data suggest either that the elevation of Nm23-H1 expression in cell lines is an artifact of cell lines, or that it occurs indirectly, for instance, if downstream of elevated RAR expression.

Estrogen was reported to elevate Nm23 expression of several breast carcinoma cell lines; we note that many metastatic breast tumors are ER-, and we failed to repeat the published trend in MCF-7 breast carcinoma cells using physiologic doses of estrogen.

Recently 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). On the basis of the MMTV-LTR data, where the cassette of transcription factors is regulated by steroids, we are investigating the effect of steroids on Nm23-H1 expression.

Several conclusions are evident to date. First, most of the agents found to elevate Nm23 expression interact with the nuclear hormone receptor family, including RAR and possibly ER and GR. Not all nuclear hormone receptor agonists are predicted to elevate Nm23 expression, as thyroid hormone reduced it (Lin *et al*., 2000). Second, all of these agents alter the expression of many other proteins, and none will be specific for Nm23. Further development will entail a search for more specific agents, and the identification of general agents with an optimal benefit/harm gene profile. Ongoing experiments are asking whether these agents elevate Nm23-H1 expression, and whether metastatic spread is limited in xenograft tumors in vivo, as a test of these first generation leads.

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