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Altered gene and protein expression by Nm23-H1 in metastasis suppression

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Abstract

Metastasis suppressor genes (MSG) are characterized by their ability to inhibit the formation of metastasis, while not affecting the growth of the primary tumor in vivo. Nm23-H1, the first MSG to be characterized, has been shown to alter both gene and protein expression in cancer cells. Recently, microarray expression profiling revealed that Nm23-H1 downregulated *EDG2*, which encodes for a lysophosphatidic acid (LPA) receptor. Reintroduction of *EDG2* into cells that express Nm23-H1 overcame the metastasis suppressive ability of Nm23-H1 in both in vivo pulmonary colonization and spontaneous metastasis assays. In addition, isotope capture affinity tag (ICAT) proteomic analysis was performed to identify differentially expressed proteins not accounted for by micro-array analysis. ICAT identified several differentially regulated proteins, including GEMIN5, a protein involved in differential mRNA splicing. The contribution of alternative mRNA splicing to cancer and cancer metastasis is poorly defined. It is possible that Nm23-H1, through the regulation of RNA processing proteins, may play a role in proteome stability.

Keywords

Nm23; Metastasis suppressor genes; Gene expression

Tumor metastasis

Metastasis is the most significant contributor to cancer-related morbidity and mortality. A simple definition of metastasis is the spread of malignant tumor cells from a primary tumor site to a secondary organ, followed by the colonization and growth of these disseminated tumor cells at the secondary organ. Despite this simple definition, the molecular and cellular events underpinning the multiple stages of the metastasis cascade are quite complex [1]. A significant number of questions regarding the mechanisms of metastasis remain, including when and how tumor cells disseminate from the primary tumor and what determines the survival of these cells at the secondary site? While the specific mechanisms at each site are not completely understood, general processes have been described [1–3]. For metastasis to ensue, the tumor cells must first acquire a motile and invasive phenotype, which allows these cells to leave the primary tumor. This step is generally followed by the invasion of tumor cells through a stromal tissue border and marked by changes in the adhesive and proteolytic abilities of the malignant cells. Following, or concurrent to this, cells invade through either the vascular endothelium or lymphatics, escaping into either the blood or

lymph vessels, respectively. Once in circulation, the malignant cells must survive the harsh environment in order to reach a distant site. Malignant cells must escape damage due to sheer forces, immune surveillance, and apoptosis induced by lack of substratum or anoikis. Once at a distant site the malignant cells lodge in a capillary bed where they adhere to the vessel walls by either changes in binding protein expression or physical size constraints. The malignant cells extravasate through the lining of blood vessel endothelial cells and basement membrane into the secondary organ, where they must adjust to the new microenvironment. To be considered a metastasis these cells must not only survive in the secondary organ as single cells but also proliferate to form metastatic colonies, which will become detectable with current imaging techniques such as MRI [4]. This entire process is very inefficient, as only a small fraction of tumor cells that enter circulation from the primary tumor will survive to form overt metastases [5].

Metastasis suppressor genes

In the past few decades, interest has grown in the relatively new field of metastasis suppressor genes (MSGs). These are genes that are functionally defined by their ability to suppress in vivo development of metastases without affecting the growth of the primary tumor. Since the identification of the first of these MSGs in 1988 [6], the number of validated MSGs has increased to over 20 [7, 8]. The majority of these MSGs have been identified by their reduced expression in metastatic cancer cells compared to congenic, non-metastatic cells using a wide variety of methods including microarray expression profiling, and subtractive library hybridization. Spontaneous metastasis assays are used to validate newly described MSGs. In these models, orthotopic transplantation of the cancer cells leads to spontaneous metastasis at distant sites. This assay incorporates primary tumor growth assessment and accurately reflects the entire metastatic cascade. Cell culture based assays, such as soft agar colony formation, wound scratch, and chemotaxis assays, have been used to quantify metastasis suppressive function in vitro, but only measure particular aspects of the metastatic process.

Nm23 metastasis suppressor gene

The *nm23* cDNA was discovered using differential colony hybridization between murine K-1735 melanoma cell lines that were all tumorigenic, but with variable in vivo meta-static potential [6]. The *nm23* mRNA levels of two cell lines with low metastatic potential were quantitatively higher than that of five related but highly metastatic cell lines. A similar pattern was observed at the protein level [9]. 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, where a general trend of reduced expression and aggressive clinical course is found (reviewed in [10]).

Transfection of Nm23 genes into highly metastatic tumor cell lines significantly reduced their tumor metastatic potential in 11 studies [11–21]. No significant effect on primary tumor formation was noted in the spontaneous metastasis experiments. These data were further validated by the characterization of an *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 [22].

Nm23 expression levels have been widely reported in many human tumor cohorts (reviewed in [10]). In these, reduced Nm23 expression has been correlated with increased metastatic potential in the majority of tumor types. This does not hold true for all cancer types, such as in neuroblastoma, where increased Nm23 expression is correlated with more aggressive disease. In these cancers a mutant *nm23* has been reported, unlike in solid tumors, such as

breast cancer, where decreased nm23 expression has not been linked to mutations in the Nm23-H1 gene [11, 23].

Several in vitro phenotypes have been reported for control- and Nm23-H1 transfected isogenic tumor cell lines. Signal responsiveness to TGF- β in colonization [11], or to IGF, serum, PDGF, LPA, etc., in motility assays, was diminished in the *nm23* transfectants [24]. A three-dimensional culture system in extracellular matrix (Matrigel) was used to study in vitro breast cell 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 [25]. The role of Nm23 in promoting differentiation was confirmed in the *Drosophila* model, with its homolog (*awd*). Loss of expression or mutation of *abnormal wing discs (awd)*, the fly homolog of Nm23, resulted in widespread abnormalities in presumptive adult epithelial tissues from the imaginal disks [26–29].

The mechanism of action of Nm23 suppression of tumor metastasis is likely complex (reviewed in [30]). At least three reported functions may contribute: (1) the histidine kinase activity of Nm23, which phosphorylates substrates such as ATP-citrate lyase [31], Aldolase C [32], and the kinase suppressor of ras [33, 34], (2) protein–protein interactions with Nm23 that may inactivate proteins and viral components that stimulate metastasis [35], and (3) gene expression alterations downstream of Nm23. This review will focus on observed changes in gene transcription- and translation-dependent on Nm23 expression, and how these alterations impact the metastasis suppressive capacity of Nm23.

Differential gene expression among control- and *nm23*-transfectants

Microarray analysis has been conducted on several sets of low- and high-Nm23-H1 expressing tumor cell lines [36]. Differentially expressed genes that varied by at least twofold in validation experiments are listed on Table 1. A much longer list of differentially expressed genes is reported in each study, without validation.

The most striking finding among these studies is the breadth of signaling proteins that are downregulated in cells expressing relatively higher levels of Nm23-H1. These include growth factors (Wnt5B), receptor tyrosine kinase inhibitors molecules (SPRY2), proteases (urokinase plasminogen activator, PLAU; MMPs-1 and -2), apoptosis inhibitory proteins (BIRC2, BCL2A1), and tumor suppressors (KLF6) in the NL9980 lung cancer cell line model [37]. In our own study, the C-100 control transfectant and H1-177 Nm23-H1 transfectant of MDA-MB-435 were analyzed for expression differences. In order to cull the long list of differentially expressed genes, two additional transfectants were profiled: S-22, which overexpresses a Nm23-H1 protein containing a Proline-96 to serine mutation, and I-205, which overexpresses a Nm23-H1 containing a Serine-120 to glycine mutation. Both mutant transfectants rendered Nm23-H1 incapable of suppressing in vitro motility [38]. Genes, which were down-regulated in the high, wild-type Nm23-H1 expressing transfectant, as compared to the C-100 vector control, and upregulated in at least one of the two mutant Nm23-H1 transfectants included growth factors (CTGF, PTN), receptor tyrosine kinases (c-MET), adhesion molecules (L1CAM), and G-protein coupled receptors (EDG2) (Fig. 1a) [36]. These expression differences were further validated at the protein level in control- and Nm23-H1-transfectants of the MDA-MB-231 breast carcinoma cell line (Fig. 1b) and in a panel of unrelated human breast cancer cell lines, classified by in vivo metastatic potential (Fig. 1c). This widespread loss of signaling is hypothesized to contribute to Nm23-H1's suppression of tumor motility and metastasis.

EDG2 studies

EDG2 is a G-protein coupled receptor for lysophosphatidic acid (LPA), a potent motility-inducing factor, which is a major component of serum. It can be converted from lysophosphatidylcholine to LPA by the enzyme autotaxin (ATX) [39]. LPA and its cognate receptors, EDG2, EDG4, EDG7, and the non-homologous GPR23 have been reported to prompt aspects of tumorigenesis and metastasis in a number of systems, particularly ovarian cancer [40–42]. LPA activity was detected at significantly higher levels in malignant effusions of ovarian cancer patients and exogenously supplied LPA enhanced metastasis incidence in an orthotopic model of ovarian cancer.

As shown on Fig. 1, EDG2 expression levels were reduced in tumor cell lines expressing wild-type Nm23-H1, but not the two mutations incapable of suppressing tumor cell motility. In addition, *EDG2* mRNA expression levels were determined in two published microarray cohorts of human breast carcinomas [43, 44]. When the cohorts were separated into high and low *Nm23-H1* expressing sets, *EDG2* expression inversely correlated with *Nm23-H1* ($P = 0.035$) [36]. Finally, an inverse correlation was also observed by immunohistochemical staining for EDG2 and Nm23-M1 in hepatocellular carcinoma tissues from wild-type and Nm23-M1 null mice [36].

In order to determine if any of the genes downregulated by wild-type Nm23-H1 functionally contributed to its suppression of motility, we asked if restoration of their expression in H1-177 cells could restore motility to serum in vitro. H1-177 cells were transiently transfected with *CTGF*, *EDG2*, *MMP2*, *c-MET*, *LICAM*, *PTN*, *FZD*, *SMO*, or a vector control and motility determined in Boyden chamber assays. Only *EDG2* overcame Nm23-H1 suppression of motility in this experiment, indicating an extraordinary selectivity in signaling (Fig. 2). Its close homolog, EDG4, which also mediates LPA signaling, was tested in the same model system and was less potent in motility induction, indicating selectivity within this family of LPA receptors [36] (Fig. 3).

We then asked whether EDG2 re-expression in high, wild-type Nm23-H1 expressing H1-177 cells would overcome its metastasis suppressive phenotype in vivo. Stable transfectants of H1-177 cells expressing either a vector or EDG2. When injected into the mammary fat pads of nude mice, primary tumor size did not vary significantly between transfectants. However, the incidence of pulmonary metastases was 51.9% of mice for the H1-177 vector clones, and 90.4% for the H1-177EDG2 clones ($P = 0.000024$). This restoration of metastatic ability was comparable to that of the low Nm23-H1 expressing cell line C-100, 89.3%. The median number of pulmonary metastases was 2 (range 0–26) in the H1-177 vector lines and significantly increased to 4 (range 0–68) in the H1-177 EDG2 clones ($P = 0.0035$); however, this number did not equal that of the C-100 line (10 in experiment 1, 4.5 in experiment 2) [45]. The data indicate that EDG2 re-expression can overcome many aspects of Nm23-H1 inhibition of in vivo metastasis.

Other genes regulated by nm23-H1 presumably, also functionally, contribute to Nm23-H1 suppression of metastasis and/or specific in vitro correlates. Current study is examining the role of altered c-Met expression in colonization. In addition, the mechanism behind the down regulation of EDG2 remains to be elucidated and further studies will focus on describing this regulation of EDG2 by Nm23.

Differential protein expression studies

It is well known that microarray studies fail to detect some important contributors, for instance genes which are present in equal amounts at the mRNA level but subject to distinct post-transcriptional control. For this reason, we performed an isotope capture affinity tag

(ICAT) proteomic analysis of control C-100 and H1-177, Nm23-H1 over-expressing MDA-MB-435, tumor cells [46]. Many of the genes found to be differentially expressed in the microarray experiments were also determined to be differentially expressed in this assay, including c-Met. However, the most abundant class of differentially expressed proteins between the two cell lines was not identified at all on the microarray analysis, those involved in RNA transcriptional modification. Of these differentially expressed proteins, four were validated by western blotting (ACIN1, Gemin5, PABPC1, and HNRPA2B1). In order to confirm the relevance of this class of differentially expressed proteins to metastasis, the ICAT data were compared to a similar analysis of an Ezrin induced osteosarcoma model system [47], and similar trends were identified for all proteins except ACIN1 [46]. Of these, Gemin5 exhibited the greatest difference in protein levels among the two model systems and was selected for further study.

Gemin5 participates in alternative mRNA splicing. In this process, identical pre-mRNA molecules are spliced to form distinct mature mRNAs, which may contribute to proteome diversity. Alternative splicing is regulated by canonical splicing signals defining intron-exon boundaries and a complex set of exonic splicing enhancers and splicing silencers that preferentially recruit the spliceosomal complex. This complex contains Gemin5, which facilitates binding of small nuclear RNAs to the complex in splicing.

In order to determine whether altered levels of Gemin5 contributed to mRNA splicing, C-100 cells were transfected with a vector or Gemin5. Both the C-100/H1-177 and C-100/C-100 Gemin5 pairs of transfectants were subjected to an alternative splicing microarray analysis, which quantifies levels of each alternatively spliced form of genes. Sixteen genes were identified, which were differentially spliced between C-100 and H1-177 cells (thus implicating Nm23-H1 expression levels), and where the ratio was restored by Gemin5 transfection of C-100 cells (thus implicating altered Gemin5 as the functional effector of Nm23 expression) [46]. In addition, Gemin5 transfected C-100 cells displayed a modest twofold reduction in tumor cell motility in vitro in Boyden chamber assays.

It is still unclear, how Nm23-H1 regulates Gemin5 expression levels in breast cancer. The contribution of alternative mRNA splicing to cancer and cancer metastasis is currently poorly defined, but it represents an emerging area of research. It is estimated that 74% of human genes encode transcripts that undergo splicing, and that 15% of human genetic diseases are associated with a mutation in either splice junctions or the spliceosomal apparatus [48]. Our data extends this trend to indicate that expression of critical parts of the spliceosome machinery also affects alternative splicing and tumor cell motility. Furthermore, this data suggest that the hypothesis of alternative mRNA splicing, which can regulate transcript diversity is modulated in metastasis and may contribute to metastatic instability.

Concluding remarks

The field of metastasis suppressor genes has grown dramatically throughout the past several decades. Recent insights into the ability of Nm23 to inhibit metastasis have shed light on new avenues of research and possible drug targets. The ability of EDG2 to overcome the metastasis suppressor ability of Nm23, both in vitro and in vivo makes it an attractive therapeutic target. Defining the mechanism by which Nm23-H1 transcriptionally down-regulates EDG2 remains a lingering question and an area of ongoing study. Nm23-H1's function in proteomic instability via the regulation of GEMIN5 is also an area of considerable interest.

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Fig. 1.

Differentially regulated gene expression by Nm23-H1. **a** Immunoblot analysis of C-100, H1-177, and substitution mutants of Nm23-H1, S22 and I-205. Decreased EDG2 expression was seen in the Nm23-H1 expressing cells but not the substitution mutants. **b** and **c** Nm23-H1 expression inversely correlated with MET, EDG2, SMO, and PTN expression in 231T (**b**) and MDA-MB-435 (**c**) metastatic cell lines. Adapted from [36, 45]

Fig. 2. Transfection of EDG2 restored motility in MDA-MB-435 + Nm23-H1 (H1-177) cells. **a** Immunoblot analysis to monitor gene transfection of H1-177 cells used in the motility assay. **b** Motility assay results of the H1-177 cells transfected with nine genes downregulated by Nm23-H1, CTGF, EDG2, MMP2, MET, L1CAM, PTN, FZD1, NETO2, and SMO. EDG2 transfection of the H1-177 cells restored motility to BSA and 1% FBS. Adapted from [36, 45]

Fig. 3.

Schematic depicting mRNA processing regulated by Gemin5. Alternative splicing occurs when exons of the primary gene transcript, the pre-mRNA, are separated and reconnected so as to produce alternative transcript. Gemin5, part of the survival of motor neurons complex (SMN), plays a critical role in mRNA splicing. The spliceosomal complex consists of small nuclear riboprotein particles, which contain small nuclear RNA (snRNA). Gemin5 functions as the snRNA binding protein of the SMN complex. Nm23-H1 regulates the expression of Gemin5, which can alter the transcript diversity and may contribute to metastatic instability

Table 1

Selected transcriptional profiling studies of tumor cell lines with altered Nm23 expression

Cell line	Validated differentially regulated genes	Reference
MDA-MB-435: C-100 control versus H1-177 Nm23-H1	Upregulated in Nm23 overexpressing ^a : MMP-1, $\alpha 3$ integrin upregulated in low Nm23 expressing: Her-2, ATX, BRCA2, and Fibronectin	[49]
CAL27: EGFPc1 versus EGFPc1-Nm23-H1	Upregulated in Nm23 overexpressing: None validated Upregulated in low Nm23 expressing: MMP-13	[50]
MDA-MB-435: C-100 control versus H1-177 Nm23-H1 versus S-22 and I-205 mutant Nm23-H1	Upregulated in Wild-type Nm23 overexpressing: CD44, SPP1, RSK1, Histone1H2B, and NME2 Upregulated in Low or Mutant Nm23 overexpressing: c-Met, EDG2, L1CAM, Smo, CTGF, PTN, and MMP-2,	[36]
NL9980: siRNA Nm23-H1 versus Scrambled siRNA	Upregulated in normal Nm23-H1 expressing ^b : CDC2L6, Centrin-2, CSTB, DNAJC19, E-cadherin, FZD6, KLHL12, MAP2, MICB, NME6, PDCD4, RASA2, SEC23B, STK40, and Timp-2 Upregulated in silenced Nm23-H1 expressing: WNT5B, TMEM158, STC1, SPRY2, SNA12, PLAUR, PHLDA1, NT5E, MMP-2, MMP-1, LGALS3, KLF6, KRT-19 and -15, JUN, F2RL1, F2R, EPAS1, EMP1 and -2, CXCL1, -2 and -3, CD44, BIRC2, BCL2A1, ANGPTL4, and ANGPT2	[37]

Genes, which have been validated as having differential expression, either up- or down-regulated, by Nm23-H1. The first column shows the cell line in which the study was carried out, as well as the comparisons used. The second column is the genes that were found to be changed in the study and subsequently validated

^a At least twofold differential expression in validation experiments

^b At least twofold differential expression in RT-PCR validation experiments