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The Potential Roles of *nm23* in Cancer Metastasis and Cellular Differentiation

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The majority of cancer patients succumb to the consequences of metastatic disease. A correlation of increased *nm23* expression to low metastatic potential has been established in several malignancies, based on published prognostic studies with tumour cohorts and transfection studies. Transfection of highly metastatic MDA-MB-435 human breast carcinoma cells with *nm23-H1* cDNA resulted in a significant reduction in the metastatic potential *in vivo*. These transfections also showed inhibition of colonisation and motility, as well as morphological and biosynthetic differentiation *in vitro*. The biochemical mechanism of Nm23-H1 action, as well as the identity of proteins involved in its functional biochemical pathway, are still unknown. We summarise published and recent research concerning the role of the *nm23* gene in metastasis and normal cellular differentiation.

Key words: cancer, metastasis, differentiation, *nm23*, nucleoside diphosphate kinase, phosphorylation
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INTRODUCTION

TUMOUR METASTASIS, the process by which tumour cells leave a primary tumour to colonise other sites of the body, is a major cause of death for cancer patients. The overwhelming complexity of metastatic dissemination of tumour cells can be realised by considering the steps that they must perform prior to successfully colonising a distant site. Metastasising cells must first disseminate from the primary tumour, invade the surrounding tissue, intravasate and extravasate the circulatory system, arrest, initiate angiogenesis and colonise distant sites, while evading the immune system.

The molecular regulation of the tumour metastatic process is currently ill defined, but its elucidation holds promise for the development of prognostic and therapeutic advances. In an effort to identify genetic changes associated with metastatic progression, differential colony hybridisation was performed on cDNAs derived from high and low metastatic murine K-1735 melanoma cells (reviewed in ref. [1]). The *nm23-1* cDNA was identified on the basis of its reduced expression in highly metastatic cell lines as compared with related cell lines with low metastatic potential. Subsequent work has associated reduced *nm23-1* expression with high metastatic potential in several metastatic tumour model systems, while analyses of human tumour cohorts of breast, hepatocellular, gastric, ovarian carcinoma and melanoma have generally correlated reduced *nm23* expression with poor prognosis (reviewed in ref. [1]).

Transfection of murine *nm23-1* cDNA into murine K-1735 melanoma cells, and human *nm23-H1* cDNA into human MDA-MB-435 breast carcinoma cell lines, resulted in significant reductions in metastatic potential *in vivo* and reduced the ability of the cells to migrate in response to several cytokines *in vitro* [2, 3]. Further analyses of these transfectant cell lines in other facets of the metastatic cascade has broadened our understanding of the role of *nm23* in metastasis. In this article, we review published and current data on the molecular, biochemical and cellular roles of *nm23* in cancer metastasis and normal cellular differentiation.

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ROLE OF nm23 IN TUMOUR METASTASIS AND DIFFERENTIATION

Two murine (*nm23-1* and *nm23-2*) and two human (*nm23-H1* and *nm23-H2*) *nm23* genes have been identified, each encoding ~17 kDa proteins that are 90% identical (reviewed in ref. [1]). Experimental and spontaneous metastasis assays have been used to demonstrate the functional role for *nm23* in the tumour metastatic process. Highly metastatic murine K-1735 TK melanoma cells were transfected with the murine *nm23-1* cDNA and empty vector as control. The *in vivo* experimental (tail vein injection) and spontaneous (subcutaneous injection) metastatic potential of the *nm23-1* and control transfected cells were determined [2]. In both assays, the *nm23-1* transfectants produced 50–90% fewer metastases than did the control transfectants. Expression of *nm23-1* did not correlate with a consistent decrease in anchorage-dependent or independent growth rates, although the *nm23-1* transfectants exhibited an altered response to the cytokine TGF-β in soft agar colonisation assays. Several studies have demonstrated that metastatically competent tumour cells are often stimulated by TGF-β in colonisation assays, while non-metastatic tumour cells are unresponsive or even inhibited by this cytokine ([4] and references therein). In agreement with these studies, we observed that control transfectants were stimulated by TGF-β in a dose-dependent manner, while *nm23-1* transfectants exhibited no significant response [2].

In a recent independent study, human *nm23-H1* cDNA was transfected into B16F10 malignant murine melanoma cell lines.

The transfected melanoma cells showed a significant reduction in invasive and metastatic potential *in vivo*, thus corroborating previously published *nm23* transfection data [5].

Several lines of evidence suggest that *nm23* may participate in the normal development and differentiation process. The *Drosophila* abnormal wing discs (*awd*) gene is 77% identical and 96% homologous in predicted amino acid sequence to Nm23. Interestingly, the *awd* gene regulates cell morphology and differentiation of the brain, proventriculus, ovaries and presumptive adult tissues in the imaginal discs postmetamorphosis ([6] and references therein). Transformation of the mutant *awd* germ line with the wild type *awd* gene restored normal development and differentiation [7].

Immunohistochemical staining of Nm23 protein in developing murine tissues also revealed a correlation of *nm23* expression with development and differentiation. The anti-Nm23 peptide 11 antibody was used to determine *nm23* expression patterns through each day of mouse embryonic development. Low and uniform levels of Nm23 protein were present throughout day 10 of development. At the onset of organogenesis, increased Nm23 protein levels were observed in the heart, brain, and all embryonic epithelial tissues, except the lungs, coincident with their functional differentiation [8]. In the mammary gland, where differentiation occurs after birth, *nm23* expression was low in the presumptive mammary bud and increased in the nulliparous and pregnant adult mammary gland. Nm23 protein levels are maintained throughout adult life by most tissues, with the exception of intestinal epithelia, where the increase is transient, and adult mammary tissue of pregnancy and lactating mice, where Nm23 expression is cyclic [8]. The phenotypic similarity of embryonic cells and tumour cells has been widely noted, suggesting the hypothesis that genes such as *nm23* may underlie both processes.

nm23 EXPRESSION IN HUMAN CANCER

Considerable evidence indicates that reduced *nm23* expression may be relevant to metastatic progression of several human cancers. In human cohorts of breast, hepatocellular, ovarian and gastric carcinoma and melanoma, reduced *nm23* expression has generally been correlated with indicators of increased metastatic potential, such as reduced patient survival and/or aggressive histopathological criteria (see Table 1 in ref. [1]). In breast carcinoma, numerous studies have reported that decreased *nm23* expression, both RNA and protein, correlate with nodal status, histological differentiation of the tumour and both disease-free and overall patient survival (reviewed in refs [1] and [9]). Two reports from one laboratory have presented data which contradict the preceding independent studies [10, 11], but the specificity of the antibody used in these reports has been questioned [9].

In neuroblastoma and pancreatic carcinoma, disease progression has been associated with increased *nm23* expression (see Table 1 in ref. [1]). It should be noted that in two independent studies mutations have been detected in *nm23-H1* and *nm23-H2* in advanced stage neuroblastoma [12, 13]. Mutations have also been reported in, and found to associate with, metastasis of colorectal carcinoma [14]. The detection of mutations in *nm23* sequences raises the intriguing possibility that mechanisms other than its reduced expression may be involved in deregulation of pathways regulated by Nm23.

The heterogeneity of the tumour metastatic process is further demonstrated in other tumour types (such a lung, thyroid and colorectal carcinoma), where no correlation between disease progression and *nm23* expression was evident (see Table 1 in ref. [1]). Several studies have reported that colorectal carcinoma expresses *nm23* mRNA and/or protein at levels higher than that of the corresponding adjacent normal colonic mucosa. In initial studies, no significant trend was observed when *nm23-H1* mRNA expression was correlated with the presence of distant metastases, although high levels of *nm23-H1* expression were associated with local aggressive behaviour [15, 16]. However, expression of *nm23* was observed to be significantly lower in colorectal carcinomas associated with liver metastasis (but still higher than in the adjacent normal colonic mucosa) than in lesions not associated with liver metastasis [17]. *nm23* expression was also found to be lower in metastatic lesions of lung and liver, but not lymph nodes, when compared with their primary tumour [18]. These reports raise the possibility that decreases in *nm23* expression may be relevant to colorectal carcinoma progression, but may be operative on a subset of primary tumours, or at the level of metastases. Further studies on larger cohorts using protein and DNA sequence analysis will be needed to clarify this issue.

Both *nm23-H1* and *H2* have been localised to chromosome 17q21, a region frequently deleted in colon cancers. While allelic deletion of *nm23* may play a role in colorectal carcinoma progression, it appears not to be a major event. When specifically examined, allelic loss of *nm23* was found to be a rare event [19, 20].

In summary, independent *nm23* RNA and protein expression studies indicate a significant correlation between low *nm23* expression and tumour aggressiveness in some tumour types. While all these studies suggest that *nm23* may be relevant to the biology of cancer metastasis, they do not prove *nm23* expression to be an independent prognostic factor.

TRANSFECTION OF THE HUMAN *nm23-H1* cDNA INTO METASTATIC HUMAN BREAST CARCINOMA CELLS

In order to ascertain whether the association of low *nm23* expression and a more aggressive phenotype in breast cancer was correlated, or rather a cause-and-effect relationship, the human *nm23-H1* cDNA was transfected into the human MDA-MB-435 breast carcinoma cell line [3]. The MDA-MB-435 breast carcinoma cell line is particularly suited for these studies, as this cell line not only expresses relatively low levels of *nm23-H1*, but it also readily metastasises upon injection into the mammary fat pad of nude mice, providing an *in vivo* breast cancer metastasis assay which closely resembles the process of human breast cancer disease. Following this mouse model, a relatively small number of tumour cells (10^5) are injected into the fourth mammary fat pad. Over a period of 3–4 months, a primary tumour develops that metastasises to organs which are major sites of human breast cancer metastasis, such as the draining lymph nodes and lungs. Metastases to other organs such as the bone, brain, and abdominal organs occasionally occur.

Overexpression of *nm23-H1* cDNA in MDA-MB-435 cells resulted in no significant difference in primary tumour size in the mammary fat pad. Control transfectants (vector without the *nm23-H1* cDNA insert) produced lymph node or pulmonary metastases in 50–59% of animals injected, while 4-fold and 9-fold overexpression of *nm23-H1* by the H1-170 and H1-177 cell lines reduced the percentage of mice with metastases to 19 and 5%, respectively [3]. Thus, *nm23-H1* has been demonstrated to possess metastasis suppressive activity in a human breast carcinoma cell line. In the same nude mouse model, *nm23-*

H2 bulk transfected MDA-MB-435 cells failed to exhibit a significant reduction in the metastatic potential relative to controls and *nm23-H1* transfected cells [3]. In addition, there seems to be no correlation between the reduced expression of *nm23-H2* and human breast cancer metastasis [21, 22]. Hence, the two human *nm23* genes may not have the same function in all cells.

To understand which steps of metastatic progression were modulated by *nm23-H1* expression, *in vitro* assays of tumour cell behaviour were performed using both the MDA-MB-435 breast carcinoma cell line and murine K-1735 TK melanoma cell line model systems. No consistent differences in growth rate, adhesiveness or protease production were observed between control and *nm23* transfected cells ([2, 3] and unpublished results). Unstimulated motility in Boyden chamber assays were comparable between control and *nm23* transfected cells. However, motility stimulated by serum, insulin growth factor (IGF) and platelet derived growth factor (PDGF) was significantly greater in control transfectants than in *nm23* transfectants [23]. Another aspect of metastatic progression examined was the aberrant colonisation response to TGF-β in soft agar assays. As previously mentioned, at least three studies have noted that metastatic but not non-metastatic tumour cells can exhibit a stimulatory response to TGF-β both *in vitro* and *in vivo*. In agreement with this trend, control transfected murine K-1735 melanoma cells were stimulated by TGF-β in a dose dependent manner, while the *nm23-1* transfectants exhibited no significant response to this cytokine. Expression of exogenous *nm23-H1* in MDA-MB-435 human breast carcinoma cells also show a reduced response to TGF-β in colonisation assays, confirming data from the murine K-1735 TK melanoma model system transfected with the murine *nm23-1* cDNA [2, 3]. This colonisation response may be highly relevant to both metastatic dissemination and eventual therapeutic intervention into the metastatic process. Colonisation is important at both the primary and metastatic sites. At the primary site, it is likely that cell proliferation and colonisation are regulated in part by local growth factors and tumour–cell stromal interactions. At a distant organ, where these interactions are probably absent, the tumour cell that can colonise either independently of external stimuli, or alternatively, can be stimulated by widely available factors such as TGF-β, may possess a significant survival advantage. Breast cancer metastasises primarily to the lymph nodes, lungs, bone, brain and liver. At diagnosis and surgery, approximately 7% of breast cancer patients have detectable distant metastases, while 38% exhibit "local" lymph node metastases (SEER data for the years 1981–1987). It is possible that in some of the 55% of "non-metastatic" and 38% "local" metastatic cases, undetectable micrometastases have already spread to distant sites, thus completing the metastatic process by the time of diagnosis and surgery. The initial primary tumour invasion and haematogenous circulatory aspects of the metastatic process are, therefore, completed, leaving angiogenesis and colonisation at the distant sites available for therapeutic intervention. Prevention of stimulated colonisation at the metastatic site may, therefore, constitute a clinically relevant target for antimetastatic therapy.

The effect of *nm23-H1* overexpression in MDA-MB-435 breast carcinoma cells on cellular differentiation has also been examined. When cultured within a reconstituted basement membrane of EHS matrix (matrigel), normal breast epithelial cells are induced to form duct-like structures, synthesise and deposit basement membrane components to the outside of the duct, synthesise and excrete sialomucin (a protein found in milk) to the lumen and growth arrest ([24] and references therein). Several breast tumour cell lines were unable to duplicate these events. In monolayer cultures, both control and *nm23-H1* transfectants exhibit similar culture morphologies and synthesis profiles [3, 24]. When grown in this matrigel system, control transfectants of MDA-MB-435 behaved similarly to other breast carcinoma cells tested, while the *nm23-H1* transfectant exhibited a remarkable degree of differentiation, similar to the normal breast epithelial cells [24]. The *nm23-H1* transfectants formed duct-like structures, with occasional central lumens, synthesised sialomucin, and deposited basement membrane components to the outside of the duct-like structures [24]. The data confirm a functional effect of *nm23-H1* expression on morphological and biochemical aspects of breast differentiation in this most interesting model system.

MOLECULAR AND BIOCHEMICAL FUNCTION(S) OF *nm23*

The molecular mechanism of Nm23 modulation of nonmetastatic and differentiated phenotypes is unknown. Several biochemical properties have been reported for Nm23 proteins, and they have all been the subject of considerable study and disagreement (reviewed in ref. [1]). The Nm23 proteins all have one biochemical activity in common: an *in vitro*, nonspecific, nucleoside diphosphate kinase (NDPK) activity, which transfers the 5′γ-phosphate from any nucleoside triphosphate (NTP) to any nucleoside diphosphate (NDP) via a high energy Nm23–phosphohistidine intermediate:

$$N_1TP + N_2DP = N_1DP + N_2TP.$$

NDPK activity has been implicated in the maintenance of NTP pools, regulation of proliferation, activation of small and heterotrimeric G proteins, and control of microtubule assembly, but each of these functions has been subject to intense debate (reviewed in ref. [1]).

Several lines of evidence suggest a dissociation of the NDPK activity of Nm23 from its biological effects on differentiation and metastasis. First, the killer of prune (*k-pn*) mutation of the *nm23 Drosophila* homologue *awd*, when expressed with the prune (*pn*) gene, causes all the developmental aberrations associated with the mutant *Drosophila awd* phenotype [25]. However, recombinant $Awd^{k\text{-}pn}$ protein has wild type NDPK activity [26]. Second, *nm23-1* transfectants exhibited no significant increase in total NDPK activity over control transfectants, and no changes in microtubule assembly patterns [27]. Upon subcellular fractionation, no cellular compartment was identified in the *nm23* transfectants which exhibited elevated NDPK activity consistent with its increased Nm23 levels and altered behaviour *in vivo* [27]. Third, NDPK activity of breast carcinomas failed to correlate inversely with patient development of lymph node metastases [11]. The data indicate that alterations in NDPK activity fail to correlate with biological changes in differentiation and metastatic potential. If the NDPK activity of Nm23 is responsible for its biological effects, then as yet unidentified mechanisms must be involved.

The Nm23-H2 protein has been reported to specifically bind DNA at the PuF site in the *CMYC* promoter, and to regulate *CMYC* transcription [28]. No analysis of Nm23-H1 protein was presented. The significance of these findings await further experimentation (reviewed in ref. [1]). The PuF site is found in many promoters, and *CMYC* would presumably not be the only gene transcriptionally regulated by Nm23-H2/PuF. A leucine zipper-like motif has been previously described for Nm23-H1

and Nm23-H2 proteins, but the three-dimensional structure of *Dictyostelium* and *Myxococcus* Nm23 homologues was inconsistent with this function (discussed in ref. [1]).

Our laboratory has identified a novel, reversible serine phosphorylation of Nm23 proteins, both *in vitro* and *in vivo* [29]. The Nm23 phosphohistidine intermediate in the NDPK reaction is a high energy (-7 kcal/mole), acid labile, base stable phosphoenzyme intermediate. We have observed that *in vitro* autophosphorylated recombinant (r) Nm23, as well as orthophosphate labelled Nm23 protein from murine melanoma and human breast carcinoma cells exhibited an acid-resistant, base-sensitive phosphorylation. Phosphoamino acid analysis identified serine 44 and serine 120, 122 and 125 as the sites of acid resistant phosphoamino acids. The phosphoserine linkage is a low energy bond (-2 kcal/mole). Once the -7 kcal/mole β-γ phosphate bond from an NTP, such as ATP, is broken to create a -2 kcal/mole serine–phosphate bond, there is insufficient bond energy to transfer this phosphate to an NDP. Therefore, the phosphoserine formation is thermodynamically distinct from the Nm23-NDPK high-energy phosphotransferase activity, establishing this serine autophosphorylation as a novel biochemical reaction of Nm23 proteins.

We have also observed that formation of Nm23 phosphoserine was inhibited by cAMP *in vitro* and forskolin *in vivo* [29], suggesting that the Nm23 phosphoserine pathway is regulated in the signal transduction process. These data provided the first link between Nm23 biochemistry and biology, in that Nm23 transfectants exhibited no significant reduction in unstimulated motility as compared with control transfectants, but decreased response to signals such as serum, IGF, TGF-β, and PDGF [23].

The potential relevance of Nm23 phosphoserine formation to NM23 regulation of metastasis was examined by comparing the *nm23* expression, orthophosphate labelled Nm23 phosphoserine levels, NDPK activity and metastatic potential of successive passages of control, and *nm23-1* transfected murine melanoma cell lines. In the *nm23-1* transfectants, quantitative increases in *nm23* expression, Nm23 phosphoserine formation and a decrease in metastatic potential was observed, while the NDPK activity was essentially unchanged when compared with control transfectants [29]. This study indicates that Nm23 phosphoserine formation is correlated with Nm23 regulation of the metastatic phenotype in the melanoma model system. The Nm23 phosphoserine formation has also been directly correlated with metastasis suppressive ability in the human MDA-MB-435 breast carcinoma model system (manuscript in preparation).

Hsu and associates have examined the role of *nm23* in two colon carcinoma sublines, a poorly tumorigenic cell line, HD3 and the highly tumorigenic cell line, U9 [30]. These sublines, derived from the same colon carcinoma cell line, are not only at different stages of tumour progression, but differ in their response to TGF-β. The HD3 line shows TGF-β-induced growth arrest and differentiation which corresponded with an increase in *nm23* mRNA and protein expression. In contrast, the more invasive and tumorigenic U9 cells are growth stimulated by TGF-β while no induction in *nm23* expression occurs. Using antisense (AS) oligonucleotides to *nm23* mRNA, *nm23* expression was reduced 2–8-fold in both cell lines [30]. Treatment of the HD3 cells with the AS *nm23* oligos inhibited adherence by over 95%, but was partially restored in response to TGF-β. Adherence of the U9 cells was only marginally inhibited (20%) by the AS *nm23* oligos, an inhibition that was unaltered in the presence of TGF-β. The TGF-β induced inhibition of proliferation of the HD3 cells was found to be partially eliminated in the presence of the AS *nm23* oligos while proliferation of the U9 cells was essentially unaffected by the AS *nm23* oligos.

These results indicate that Nm23 may play a role in TGF-β-induced growth inhibition of cells. How Nm23 acts in this signal transduction pathway is unclear, but evidence suggests that it is not due to Nm23-H2/PuF induced transcription of the *CMYC* gene, as induction of Nm23 mRNA by TGF-β in HD3 cells resulted in a decrease in *CMYC* expression. TGF-β-induced growth of HD3 cells correlates with activation of ras proteins and the 57 kDa MBP kinase while inhibiting the activity of the 105 and 130 kDa MBP kinases. Hsu and associates have postulated a model in which Nm23 would be a component in the *ras*-dependent signal transduction pathway upstream of the MBP kinases, a pathway that is not involved in TGF-β-induced proliferation of the more invasive and tumorigenic U9 cells [30]. While this is an intriguing possibility, further experimentation will be required before the role Nm23 plays in the TGF-β signal transduction pathway, its relevance to the progression of colorectal carcinoma and its relationship to the antimetastatic activity of Nm23 are known.

TGF-β acts as a growth inhibitor to most cells, but becomes a stimulator of both growth and invasion as cells become more metastatic [4]. The observations with the HD3 and U9 cells implicate Nm23 as being central in determining whether TGF-β (and potentially other cytokines) act to stimulate or inhibit proliferation. Thus, low *nm23* expression or loss of function would allow cells to respond in an inappropriate manner to TGF-β, potentially enabling them to proceed down the pathway leading to the fully metastatic phenotype.

FUTURE DIRECTIONS

The molecular mechanism of Nm23 regulation of differentiation and metastatic potential is still unknown, although candidate biochemical activities have been identified. Most promising among these candidate activities is the serine phosphorylation of Nm23, owing to its correlation to metastatic potential in melanoma and breast carcinoma cells. While the elevation of overall tumour cell *nm23* expression levels can be considered a target for the therapy of micrometastases for certain cancers, elevation of the functional Nm23 activity may suffice, and could thus be therapeutically useful in the absence of an overall elevation of Nm23 expression. A major research goal of our laboratory is to elucidate the biochemical mechanism of Nm23 regulation of differentiation and metastatic potential, and ultimately to use this knowledge to identify pharmaceutical agents with potential therapeutic benefits in the treatment of metastatic disease.

Known and proposed biochemical mechanisms of Nm23 action are currently under study via site-directed mutagenesis. The ability of each mutated protein to inhibit breast cancer metastasis in an *in vivo* metastasis assay is being determined. Should one of these known or proposed biochemical activities of Nm23 be found to be responsible for its metastasis inhibitory effects, the functional domain of Nm23 will be narrowed down by the production and transfection of *nm23-H1/nm23-H2* chimeric molecules.

Also unknown is what proteins Nm23 interacts with to mediate its effect on tumour metastasis and differentiation. Using several protocols that allow the identification of interacting proteins, we are currently attempting to identify proteins which interact with Nm23. Proteins which interact with Nm23-H1 may represent upstream or downstream components of known

biological pathways, in which case a biochemical function for Nm23 activity could be established.

With these data, we are attempting to identify pharmaceutical agents capable of elevating Nm23 function, or also identify agents that are preferentially cytotoxic/cytostatic to low *nm23*-expressing cells. In collaboration with the Developmental Therapeutics Programme, Division of Cancer Treatment, NCI, we have identified 45 agents that are selectively cytostatic, *in vitro*, against a panel of breast and melanoma cell lines that are low *nm23* expressors. These agents could potentially elevate *nm23* expression, its functional biochemical activity, improve the expression or function of a protein downstream on its functional biochemical pathway, or act independently of Nm23. *In vitro* experiments will distinguish between these possibilities. In addition, the effect of each agent on growth and metastatic potential will be determined both *in vitro* and *in vivo*. These experiments may lead to the identification of a novel class of pharmaceutical agents with a mechanism of action based on molecular experimentation.

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