Chapter 2

NM23H2 as a key metastasis suppressor in breast cancer
2.1. Introduction

2.1.1. Examination of previous reports of NM23H2 acting as metastasis suppressor in breast cancer

Is there a compelling rationale for considering NM23H2 as a key metastasis suppressor gene (MSG) in breast cancer progression? As an MSG, NM23H2 must show three cardinal attributes: first, its expression must be reduced in metastasis compared to localized primary tumors (human samples); second, metastatic cell lines should have lower expression of NM23H2 than non-metastatic cell lines (for example, NM23H2 transcript and protein levels should be lower in metastatic MDA-MB-231 cells compared to non-metastatic MCF-7 cells) and third, over expression of NM23H2 in metastatic breast cancer cell lines should diminish their metastatic potential (in vivo such as in a relevant immunodeficient mice model). An unbiased examination of previous reports on NM23H2 in breast cancer progression is given below.

In previous reports, NM23H2 has been implicated in breast cancer metastasis using several model systems. In vitro cell culture based model systems were used to address the role of NM23H2 in metastasis. It was reported that over expression of NM23H2 in highly metastatic human breast cancer cell line MDA-MB-231 led to suppression in cell motility in modified Boyden chamber assay. Similarly over expression of NM23H2 led to suppression in anchorage independent growth in MDA-MB-231 cells using soft agar colony formation assay (McDermott et al., 2008). These results suggested that NM23H2 could influence at least two steps of metastasis cascade. Firstly, invasion and cell motility, which is the first step of metastasis in which cells exit from the primary tumor as well as start invading nearby tissues. Secondly, anchorage independent cell proliferation which is regarded as a hallmark of cell transformation which allows cell
cycle progression and survival in the absence of cell adhesion. Another study reported that NM23H2 inhibited cell migration in a breast cancer model cell line MCF-7 after estrogen treatment (Rayner et al., 2008). Thus, NM23H2 might act as a suppressor of breast cancer metastasis by negatively regulating cancer cell motility and colony forming potential.

Study of in vivo mouse and rat models also showed that NM23H2 suppressed breast cancer metastasis. A previous report suggested that NDPK alpha (homologue of NM23H2) suppressed pulmonary metastases in spontaneous metastasis assays using rat breast cancer cell lines; almost 50% reduction in metastatic foci in lungs was observed. Interestingly NDPK beta (homologue of NM23H1) did not have any effect on pulmonary metastasis. The same study also reported that NDPK alpha (homologue of NM23H2) had higher expression in MTC cell line of rat with poor metastatic ability compared to highly metastatic MTLn3 cells (Fukuda et al., 1996).

In another study, it was reported that NM23H2 over expressing MDA-MB-435 cells seeded metastasis to lungs in orthotopic Severe Combined Immuno Deficient (SCID) mice model. The study found almost 90% less metastatic foci in lungs upon NM23H2 over expression compared to control MDA-MB-435 cells (Bhujwalla et al., 1999).

While in vitro and in vivo reports suggested that NM23H2 might act as metastasis suppressor in breast cancer, expression analysis of human tumor samples have been few, and a clear relationship between expression of NM23H2 and status of malignancy has not been observed (Tokunaga et al., 1993). Moreover, the studies so far have mostly focused on NM23H1, a paralogue of NM23H2 in human breast cancer progression (Hartsough and Steeg, 2000). Irrespective of the model systems used, previous studies
did not provide mechanistic insights. There remains plethora of unanswered questions like what are the molecular determinants of NM23H2 mediated metastases suppression, and does expression of NM23H2 associate with disease prognosis.

2.1.2. A combinatorial strategy for assessing relationship of NM23H2 to patient survival, and regulation of metastasis in vitro and in vivo

One of the aims of this study has been to thoroughly examine whether NM23H2 could act as a MSG in breast cancer progression. Keeping in view the cardinal features of a MSG, a combinatorial strategy was adopted which included (a) large scale bioinformatic analyses of human breast tumor transcriptomes, as well as analysis of expression levels of NM23H2 at mRNA and protein levels in clinical samples, (b) probing levels of NM23H2 in relevant cell lines, and (c) in vivo studies involving suitable mice models to investigate action of NM23H2 as a metastasis suppressor (Figure 2.1).

![Diagram showing the integrative approaches to probe role of NM23H2 in breast cancer metastasis.](image)

**Figure 2.1: Integrative approaches to probe role of NM23H2 in breast cancer metastasis.** Beginning with examination of breast tumor gene expression pattern through meta-analysis, as well as qPCR, the approach also included immunohistochemical analysis of NM23H2 in primary tumors and lymph node metastases from same patients, ascertaining reduced expression in metastatic and non-metastatic cell lines, and probing metastasis seeding in nude mice.
2.2. Results

2.2.1. Meta-analysis of >30 MSGs shows consistently reduced expression of NM23H2 in advanced breast tumors

Loss of MSG’s expression is linked to cancer metastases. Therefore, the study aimed to perform unbiased transcriptomic analysis of clinical cancer sample datasets available in public repositories for expression of known metastases suppressor genes. To determine the potential clinicopathological implications of altered MSG’s expression, meta-analysis was performed to measure the changes in expression of more than 30 established MSGs across four independent studies of tumor transcriptomes from breast cancer patients (n=947; Figure 2.2A). The samples were divided in two groups: advanced (comprising of stage III and stage IV), and early stages (comprising of stage I and stage II). The difference in expression for NM23H2 in advanced stages compared to early stage has been shown by box plots for each independent clinical study (Figure 2.2B). Interestingly, out of all differentially expressed MSGs only NM23H2 is significantly down regulated at transcript levels in all the four studies (Figure 2.2C). These results suggested that reduced expression of NM23H2 is associated with advanced stages.
Figure 2.2: NM23H2 expression is low in advanced breast tumors. A. Analysis of differential expression of 36 MSGs across four independent clinical cohorts of breast cancer (expression index: red, up-regulation; blue, down-regulation). B. Box plot NM23H2 showing reduced expression in advanced (stage 3 and 4) relative to early stages (stage 1 and 2). C. Heat map of 19 MSGs with reduced expression in advanced stages in at least one data set; NM23H2 being only MSG with reduced expression in all the four datasets tested (scale: fold change of expression). Color denotes expression values.

2.2.2. Higher NM23H2 level within primary tumors associated with enhanced overall patient survival

As observed earlier the expression of NM23H2 is reduced in advanced stages of tumor progression (Figure 2.3A-C). It could be speculated that loss of expression of NM23H2 in the primary tumor could be one of the contributing factors for metastasis. Thus,
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reduced expression of NM23H2 could potentiate a primary tumor for seeding of metastases. As metastasis leads to marked reduction in patient survival, this study probed how does the level of NM23H2 within their primary tumors associate with patient survival.

A total of 728 patient derived breast tumor transcriptomes along with their related patient information from three independent datasets were used to assess the association of NM23H2 with overall patient survival. In the first study (Hatzis et al., 2011), expression of NM23H2 across tumors of 461 patients was divided into two sets: high versus low (n=230 and 231, respectively). Kaplan–Meier survival analyses revealed that patients whose primary tumors displayed low expression of NM23H2 had less probability to survive; conversely the patients with high expression of NM23H2 in their primary tumors had higher survival probability (Figure 2.3A). These results indicated that NM23H2 level could be used to stratify early stages to predict clinical outcome. Further, analysis of tumor and survival data from an independent set of 167 patients (Hu et al., 2006) showed similar trend (Figure 2.3B), i.e. higher NM23H2 level in primary tumor significantly correlated with increased survival. In an additional dataset of 100 patients (Ma et al., 2004) ranking of tumors based on NM23H2 expression showed that patients with higher NM23H2 within tumors had increased survival (Figure 2.3C). Taken together, these results from independent datasets showed that NM23H2 level within primary breast tumors associated with their malignant spread and subsequent clinical outcome. Searching for ways of increasing levels of NM23H2 in primary tumors might be of potential therapeutic intervention.
Figure 2.3: Higher expression of NM23H2 associated with survival in breast cancer patients. Kaplan-Meir method was used to analyze the relationship between expression of NM23H2 expression within tumors and survival of 728 patients. (A) Data from Hatzis C et al. 2011, n=461, (B) Hu Z et al. 2006, n=167, and (C) Ma XJ et al. 2004, n=100. Transcript level of NM23H2 in the two groups tested shown as box plot in right panel; statistical significance was calculated using Student t-test.
2.2.3. NM23H2 levels are decreased in advanced breast tumors; qPCR analysis of patient tumors

In order to quantify the extent of deregulation, NM23H2 expression was specifically examined in pathologist certified commercially available primary and metastatic tumors from patient samples. Quantitative real time PCR (qPCR) for NM23H2 showed significantly lower NM23H2 transcript level in advanced stages of breast cancer tissues (Figure 2.4; n=30, ~1.7 fold depletion; P<0.003) confirming the trend observed in gene expression meta-analyses.

![Graph showing decreased expression of NM23H2 in metastatic tumors](image)

**Figure 2.4**: Decreased expression of NM23H2 in metastasis compared to primary tumor from patients. qPCR for NM23H2 to detect its transcript in 30 tumors from breast cancer patients showed depleted NM23H2 in metastatic tumors. Analysis of primary tumor and metastases revealed reduction in NM23H2 expression in metastases.

2.2.4. NM23H2 expression is reduced in autologous lymph node metastasis

As a classic metastasis suppressor, expression of NM23H2 should be reduced in metastasis compared to the corresponding primary tumors. This was probed using immunohistochemical analysis of NM23H2 in primary breast tumors (n=40) and the autologous (derived from same patient) lymph node metastasis (n=40). The analysis of
matched tumor samples showed reduced NM23H2 staining in metastasis confirming decreased expression during metastatic progression of breast cancers (Figure 2.5).

**Figure 2.5: Decreased expression of NM23H2 in metastasis compared to primary tumor from the same patients.** Representative immunohistochemistry images for NM23H2 in primary breast tumors and autologous lymph node metastasis (left panel). Quantification of immunostaining for NM23H2 (right panel). Statistical significance calculated by Pearson chi-square test.

### 2.2.5. Differential expression of NM23H2 in breast cancer cell lines that differ in metastatic potential

Based on analyses above which showed decreased NM23H2 expression in advanced cancers, it was next investigated whether the expression of NM23H2 in relevant breast cancer cell lines mimicked the pattern observed in early and advanced cancer stages. The study made use of two cell lines: widely used MDA-MB-231 cells which are highly aggressive and metastatic and MCF-7 cells which are non-aggressive and poorly metastatic. *In vitro* invasive potential of these two cell lines was checked by Boyden chamber assay. The inner chamber called insert was coated with thin layer of matrigel basement matrix. Those cells which have the ability to degrade this layer of matrigel could migrate towards the other side of the insert. The MDA-MB-231 cells which are...
highly metastatic in nature had several fold higher invasive potential through matrigel basement matrix compared to MCF-7 cells which are poorly metastatic (Figure 2.6A). The protein level of NM23H2 was found to be higher in MCF-7 cells (poorly metastatic) compared to MDA-MB-231 cells (highly metastatic) (Figure 2.6B). These results corroborated our previous findings that aggressive tumors have low NM23H2 levels.

![Figure 2.6: Decreased expression of NM23H2 in metastatic breast cancer cells (MDA-MB-231) compared to non metastatic breast cancer cells (MCF-7). A. Invasion assay to reconfirm that MDA-MB-231 cells are highly invasive compared to MCF-7 cells. B. Western blot analysis to investigate NM23H2 expression in both the cell lines (Lower band belongs to NM23H2 while upper band belongs to NM23H1 as antibody used non specifically picks NM23H1 alongside NM23H2).](image)

2.2.6 Generation and validation of breast cancer cells with stably increased expression of NM23H2

To study the mechanisms of NM23H2 mediated regulation of metastasis, two variants of MDA-MB-231 cells lines were generated; MDA-MB-231 cells with over expression of NM23H2 tagged with GFP and MDA-MB-231 cells with over expression of GFP as control cells. In brief, MDA-MB-231 cells were transfected with plasmid containing NM23H2 cDNA tagged with GFP and empty vector expressing only GFP. After 48
hours post transfection, stable cells were selected by treating it with antibiotic G418 sodium salt (.50 mg/ml). A detailed step by step information regarding generation of stable cell line is discussed in the materials and methods section. Antibiotic selected stable cells were pooled and over expression were confirmed by qPCR (Figure 2.7A) and western blotting (Figure 2.7B).

**Figure 2.7: Generation of suitable cell culture based model system to study the role of NM23H2 in breast cancer.** **A.** Stable over expression of NM23H2 in MDA-MB-231 cells was confirmed by qPCR at transcript level. **B.** Stable over expression at protein level was confirmed by western blotting.

2.2.7. **NM23H2 over expressing cells have reduced invasive potential through basement membrane matrix**

Invasion is a crucial step of metastasis in which cells from primary tumors detach themselves and start invading nearby tissues by breaking the basement membrane and degrading the extracellular matrix. Once stable NM23H2 over expressing cells were generated, the effect of increased level of NM23H2 on invasion of cancer cell lines was probed using *in vitro* Boyden chamber assay. NM23H2 over expression led to reduced invasion in highly metastatic MDA-MB-231 cells. This result suggested that NM23H2 might inhibit metastasis by inhibiting invasion (Figure 2.8).
2.2.8. NM23H2 over expressing cells have reduced trans-endothelial migration potential

Metastases to distant sites depend on the ability of invading cancer cells to break endothelium barrier of blood vessels by a process called intravasation. This step is critical for seeding metastases to distant organs. Therefore, the effect of NM23H2 over expression on this particular key step of metastasis was investigated. A modified Boyden chamber to mimic this condition in-vitro was used; uniform monolayer of Human Umbilical Vein Endothelial Cells (HUVEC) was grown in the inner chamber which served as an endothelial barrier. NM23H2 over expression led to reduced trans-endothelial migration in highly metastatic MDA-MB-231 cells (Figure 2.9). This result showed that NM23H2 might regulate intravasation step of metastatic cascade.
Figure 2.9: NM23H2 suppresses trans-endothelial migration *in-vitro*. MDA-MB-231 cells with stable expression of NM23H2 show decreased efficiency in crossing an endothelial cell layer.

2.2.9. NM23H2 suppresses metastasis *in vivo*

The MDA-MB-231 cells with stable expression of NM23H2 were used for in vivo investigation of tumor metastasis. Over expression of NM23H2 significantly decreased the size of metastatic nodules of MDA-MB-231 breast cancer cells in lung compared with GFP only control cells, as detected under a fluorescent microscope (Figure 2.10A). Hematoxylin and Eosin (H and E) staining (Figure 2.10B) showed that significantly less space in pulmonary alveolus was occupied by NM23H2 over expressing breast cancer cells after 8 weeks post-injection compared with the controls. Decreased numbers of metastatic nodes in lung after tail vein injection was quantified (Table 2.1). The results further confirmed that over expression of NM23H2 in tumor cells inhibited the formation of metastatic nodules in lung.
Figure 2.10: NM23H2 inhibits metastasis in vivo. A. Fluorescence microscopy images showing colonization in lungs (false colour denotes metastatic cancer cells). B. H&E staining in lung cryo-sections showing metastatic lesions in mice injected with vector whereas no lesions are found in NM23H2 over expressing MDA-MB-231 cells.

Table 2.1. Quantification of metastatic foci in lungs of nude mice shows that NM23H2 is suppressor of metastasis in vivo.

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<tr>
<th>Animal No.</th>
<th>Metastatic nodules (Vector)</th>
<th>Metastatic nodules (NM23H2 over expressing cells)</th>
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2.3. Discussion

2.3.1. Integrative approaches support a role for NM23H2 in breast tumor metastasis suppression

Large scale analyses of patient derived breast tumors as described above clearly suggested a role for NM23H2 in regulation of metastatic potential of breast tumors. While previous studies evaluated anti-metastatic functions of NM23H2 using \textit{in vitro} and \textit{in vivo} models, the present study used human tumor samples exhaustively. Analysis of NM23H2 expression in clinically certified breast tumor specimen cleared showed an association between reduced level of NM23H2 and progressively higher grade of malignancy. Furthermore, level of NM23H2 in a primary tumor associated with patient survival; while lower NM23H2 expression correlated with worse survival, higher NM23H2 in a primary tumor correlated with better survival.

Given that level of NM23H2 could be important for metastatic outcome, it is interesting to note that several agents can enhance the expression of NM23H2. Some of the agents include: Estrogen (17 beta-estradiol) or estrogen receptor (ER) beta selective agonists such as diarylpropionitrile, medroxyprogesterone acetate (MPA), and progesterone (Ouatas et al., 2003; Rayner et al., 2007; Syed et al., 2005). Given that breast cancer progression is significantly influenced by estrogen and progesterone receptor status, it is reasonable to speculate that NM23H2 could be involved in signaling pathways downstream of estrogen and progesterone.

2.3.2. A case for gene expression regulatory role of NM23H2 in control of breast cancer metastasis

Previous report from this laboratory has shown that NM23H2 regulates proto oncogene c-MYC in lung cancer A549 cells (Thakur et al., 2009). This suggested gene expression
regulatory roles of NM23H2 could be decisive in control of metastatic progression of
tumors. Recently c-MYC has been shown to regulate breast cancer metastases (Liu et al., 2012). However, no studies have examined gene expression program of cancer cells
with altered level of NM23H2. Importantly, the prospect of a broad NM23H2-
dependent gene expression regulatory network opposing metastasis has not been
evaluated. In this context, cell lines generated in this study could be used for probing
how increased expression of NM23H2 induces gene expression changes and how these
changes, in turn, contribute to breast tumor metastasis.

2.4. Materials and Methods

2.4.1. Cells and culture conditions

MDA-MB-231 and MCF-7 cells were obtained from the national repository of cell lines
at National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco’s
modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37°C
in 5% CO₂ environment.

2.4.2. Western Blotting

Immunoblotting was performed as follows; cells were harvested and lysed with 1X cell
culture lysis reagent (CCLR) (Promega, USA). Protein concentrations were determined
with the BCA protein assay kit (Pierce, USA). Protein samples (50-60μg) were
separated by SDS-PAGE, transferred onto an Immobilon membrane (Millipore, USA),
and blotted with antibodies against NM23H2 (Abcam, USA), Turbo GFP (Evrogen)
and Beta Actin (Sigma). ALP conjugated secondary antibody (Sigma) and BCIP-NBT
(Sigma) was used as its substrate.
2.4.3. Meta-analyses of patient samples

For meta-analysis of NM23H2 expression across breast cancer tumors, transcriptome datasets were used from Expression Project for Oncology (expO) database (GSE2109), GSE25066 (Hatzis et al., 2011), GSE10510 (Calabro et al., 2009) and GSE5847 (Boersma et al., 2008). ExpO is hosted by International Genomics Consortium (IGC, USA, www.intgen.org) and provides gene expression datasets of clinically annotated sets of tumor samples. Gene expressions across datasets were normalized using Z score transformation (Cheadle et al., 2003). Normalized expression levels of NM23H2 were checked among three groups. Significance of differential expression was calculated by student t-test.

2.4.4. Survival analysis of patient samples

Expression of NM23H2 across tumors of all patients was divided into two sets: high versus low. Survival analysis was performed by drawing Kaplan-Meir curves using information on tumor expression of NM23H2 and duration of survival of patients. The data were obtained from GSE25066 (Hatzis et al., 2011), GSE1992 (Hu et al., 2006), and GSE 1378, GSE1379 (Ma et al., 2004).

2.4.5. Generation of MDA-MB-231 cells stably over expressing for NM23H2 protein

MDA-MB-231 cells were obtained from the national repository of cell lines at National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO₂ environment. MDA-MB-231 cells were transfected using pCMV-AC-GFP (Origene Inc.) plasmid vector containing a CMV promoter that regulated expression of NM23H2 protein, and stable clones were G418 selected. Transcript level of NM23H2 was measured by quantitative real time qPCR using SYBR green chemistry on ABI 7500

2.4.6. Cell invasion and trans-endothelial migration assays

Invasion potential of cells and trans-endothelial migration was determined by a variation of Boyden chamber assay using BD biosciences invasion chamber, and Cell Biolabs CytoSelectTM transendothelial migration assay kit respectively as per manufacturer’s instructions. The upper surface of the insert membrane was pre-coated with a uniform layer of dried basement membrane matrix solution containing extracellular matrix extracts isolated from mouse EHS sarcoma tissue with laminin as a major component, collagen type IV, heparan sulfate proteoglycan, entactin and other minor components. Cells were suspended in medium without serum or growth factors, and medium supplemented with serum was used as a chemo attractant in the lower chamber. After incubation at 37°C for 24 h, the top chambers were wiped with cotton wool to remove the noninvasive cells. The invading cells on the underside of the membrane were fixed in 100% methanol for 10 min, air dried, stained in 0.1% crystal violet. Cells were lysed and quantified using colorimetric assay by taking OD at 562 nm. Similarly for trans-endothelial migration assay before putting cancer cells into the chamber, uniform monolayer of Human Umbilical Vein Endothelial Cells (HUVEC) was grown in the inner chamber. The mean of triplicate assays for each experimental condition was used.

2.4.7. Clinical annotation of tumor specimen analyzed

Expression of NM23H2 was checked in commercially available breast tumor samples (cDNA qPCR arrays BCRT02 from Origene, Inc. USA) by quantitative real time PCR using SYBR green chemistry on ABI 7500 using the primers described before for
NM23H2. All samples were duly certified by qualified pathologists. The details on age, sex and pathology of patients from whom primary breast tumors and matched lymph node metastasis were used for analysis of NM23H2 are described in appendix 1.

2.4.8. Immunohistochemical analysis

For Immunohistochemistry, primary breast tumor and autologous lymph node metastases were stained for desired protein (NM23H2) and counterstained for haematoxylin. Antibodies against NM23H2 were from Kamiya Biomedical Company, Seattle, USA. Scoring was done for no staining, moderate staining and high staining.

2.4.9. Tail vein metastases assay

For experimental metastasis, 2 million cells were washed and resuspended in PBS. Five-week-old nude mice (BALB/C-nu/nu) were injected into the lateral tail vein, and the animals were maintained in a sterile animal facility. Each tumor cell subline was injected into ten mice. After ten weeks, the mice were sacrificed, and the lungs were examined for metastases. Lungs were fixed and images were taken in fluorescent microscope for GFP. Lung sections were stained for H&E (Haematoxylin and Eosin) and examined. All animal experiments were done in compliance with animal ethics committee, Mayo Clinic, Rochester, USA.

2.4.10. Statistical analysis

In vitro cellular assays were done at least in triplicate and p-values were derived using Student’s t-test unless stated otherwise. The error bars represented standard deviation unless described otherwise. Statistical significance for immuno-staining of NM23H2 in primary tumors and autologous lymph node metastases was calculated using Pearson chi-square test.