Nm23-H1 Suppresses Metastasis by Inhibiting Expression of the Lysophosphatidic Acid Receptor EDG2

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Abstract

Nm23-H1 transcriptionally down-regulates expression of the lysophosphatidic acid receptor EDG2 and this down-regulation is critical for Nm23-H1–mediated motility suppression in vitro. We investigated the effect of altered EDG2 expression on Nm23-H1–mediated metastasis suppression in vivo. Clonal MDA-MB-435–derived tumor cell lines transfected with Nm23-H1 together with either a vector control or EDG2 had similar anchorage-dependent and anchorage-independent growth rates in vitro. However, a 45- and 300-fold inhibition of motility and invasion (P < 0.0001), respectively, was observed in Nm23-H1/vector lines, whereas coexpression of EDG2 restored activity to levels observed in the parental line. Using fluorescently labeled cells and ex vivo microscopy, the capacity of these cells to adhere, arrest, extravasate, and survive in the murine lung over a 24-h time course was measured. Only 5% of Nm23-H1/vector–transfected cells were retained in the murine lung 6 h following tail vein injection; coexpression of EDG2 enhanced motility to Nm23-H1/vector lines (P < 0.01). In a spontaneous metastasis assay, the primary tumor size of Nm23-H1/vector and Nm23-H1/EDG2 clones was not significantly different. However, restoration of EDG2 expression augmented the incidence of pulmonary metastasis from 51.9% to 90.4% (P = 2.4 × 10−5), comparable with parental MDA-MB-435 cells. To determine the relevance of this model system to human breast cancer, a cohort of breast carcinomas was evaluated. In addition, expression of autotaxin, the enzyme that metabolizes LPA and its cognate receptors, EDG2, EDG4, EDG7, and nonhomologous GPR23, have been shown to prompt aspects of tumorigenesis and metastatic outgrowth. LPA activity was detected at significantly higher levels in malignant effusions of ovarian cancer patients and exogenously supplied LPA enhanced metastatic incidence in an orthotopic model of ovarian cancer (21–24). In addition, expression of autotaxin, the enzyme that metabolizes lysophosphatidylcholine to produce LPA, was higher in hepatocellular carcinoma tissue compared with normal liver specimens and overexpression of this phospholipase augmented the invasive, angiogenic, and metastatic properties of Ras-transformed fibroblasts in mice (25). EDG4 expression correlated positively with ovarian tumor growth and EDG2 expression was linked to the motility and invasiveness of several metastatic cell lines (26, 27). In an in vivo model, EDG2 activity promoted both primary tumor growth and osteolytic bone metastasis of the breast carcinoma cell line MDA-B02 (28, 29).

Introduction

The study of metastasis suppressor gene function has provided a genetically tractable system for which to study the molecular basis of the complex, therapeutically important process of metastasis. The metastasis suppressor Nm23-H1 inhibits multiple metastatic correlates. Overexpression of Nm23-H1 in tumor cells nearly abolishes tumor cell motility and invasion, prompts cellular differentiation, and inhibits anchorage-independent growth and adhesion to fibronectin, laminin, and vascular endothelial cells (1–11). One or more of these suppressive phenotypes may contribute to its capacity to inhibit metastasis in vivo. Nm23-H1, or its mouse or rat orthologues, has been shown to suppress metastatic incidence and/or the number of metastatic lesions in at least 10 independent, in vivo models of metastasis (7, 10–18). Transfection of Nm23-H1 suppressed metastatic incidence from 40% to 98% in spontaneous metastasis assays without affecting primary tumor size (7, 10, 11). Additionally, mice lacking expression of the murine homologue of Nm23-H1, Nm23-M1, have a ~2-fold enhancement in the incidence of pulmonary metastases when induced to form hepatocellular carcinoma (19).

The molecular mechanism of action of Nm23-H1 has not been well described but was recently shown to transcriptionally reduce the expression of the lysophosphatidic acid (LPA) receptor gene EDG2 in several metastatic cell lines (20). This diminished expression was shown to be critical for Nm23-H1 motility suppression as reexpression of EDG2 enhanced the motility of Nm23-H1–suppressed cells by 60-fold and silencing EDG2 inhibited metastatic cell motility by >70% (20). Eight other genes transcriptionally down-regulated by Nm23-H1 and encoding cell surface receptors and/or secreted factors had almost no capacity to restore motility to Nm23-H1–suppressed tumor cells.

LPA and its cognate receptors, EDG2, EDG4, EDG7, and nonhomologous GPR23, have been shown to prompt aspects of tumorigenesis and metastatic outgrowth. LPA activity was detected at significantly higher levels in malignant effusions of ovarian cancer patients and exogenously supplied LPA enhanced metastatic incidence in an orthotopic model of ovarian cancer (21–24). In addition, expression of autotaxin, the enzyme that metabolizes lysophosphatidylcholine to produce LPA, was higher in hepatocellular carcinoma tissue compared with normal liver specimens and overexpression of this phospholipase augmented the invasive, angiogenic, and metastatic properties of Ras-transformed fibroblasts in mice (25). EDG4 expression correlated positively with ovarian tumor growth and EDG2 expression was linked to the motility and invasiveness of several metastatic cell lines (26, 27). In an in vivo model, EDG2 activity promoted both primary tumor growth and osteolytic bone metastasis of the breast carcinoma cell line MDA-B02 (28, 29).

Nm23-H1 and EDG2 play opposing roles in the metastatic process and Nm23-H1 has been shown to antagonize the expression of EDG2. Therefore, we hypothesized that forced expression of EDG2 in Nm23-H1–suppressed MDA-MB-435 cells would restore a metastatic propensity to these cells. Whereas
exogenous expression of Nm23-H1 alone nearly abolished cell motility and invasion, forced expression of EDG2 fully restored a motile, invasive, and adhesive phenotype. EDG2 expression also enhanced tumor cell retention in the murine lung, antagonizing the effect of Nm23-H1 expression alone, and overcame Nm23-H1 metastasis suppression in an in vivo model of spontaneous metastasis.

Materials and Methods

Cell culture techniques and stable transfection of EDG2. Cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% (v/v) penicillin/streptomycin (Invitrogen) in an atmosphere of 5% CO2 at 37°C and grown to ~85% confluency before harvesting for experiments. Human MDA-MB-435-derived cell lines C-100 and H1-177 were described previously (32). H1-177 cells were clonally selected to express EDG2. The EDG2 coding sequence was subcloned from pCDNA3.1 into pCDNA3_Zeo (Invitrogen) with standard cloning techniques and sequence verified. H1-177 cells were transfected with either pCDNA3-Zeo empty vector or containing EDG2 using standard Effectene (Qiagen) procedures. Cells were clonally selected in 100 μg/mL zeocin medium for 28 days. Clones were screened for EDG2 expression by immunoblotting.

Cell lysis and immunoblotting. Lysates were prepared as described (31) and protein concentration was determined by a bicinchoninic acid protein assay ( Pierce). Equivalent amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham Biosciences). Membranes were probed with anti-Nm23-H1 (BD Biosciences), anti-EDG2 ( Abcam), and anti-tubulin (Oncogene). Protein-antibody complexes were visualized with horseradish peroxidase–conjugated secondary antibodies and LumiGLO (Cell Signaling Technology). Relative protein expression was determined by spot densitometry.

Cell motility and invasion assays. Cell migration assays were performed as described previously (32). Lower wells contained 0.1% bovine serum albumin (BSA) DMEM with or without 1% FBS or LPA (Sigma). Cells were added to each upper well in 0.1% BSA DMEM and incubated 4 h in a humidified chamber at 37°C. H1-177 cells were transfected with either pCDNA3-Zeo empty vector or containing EDG2 using standard Effectene (Qiagen) procedures. Cells were clonally selected in 100 μg/mL zeocin medium for 28 days. Clones were screened for EDG2 expression by immunoblotting.

Cell viability assay. Each cell line was seeded in five wells in four replicate 96-well plates at 2.5 × 10³ per well. Cell viability was assayed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 24, 48, 72, and 96 h after seeding. MTT reagent (Sigma) was added to each well to a final concentration of 0.5 μg/mL and incubated at 37°C, 5% CO₂ for 4 h. Following the incubation, cells were solubilized in DMSO. Color development proceeded for 1 h at 37°C and cell number was estimated by absorbance at 570 nm using a VersaMax microplate reader. Absorbance readings from empty wells were background subtracted. Mean absorbance was calculated and graphed in Microsoft Excel. Each assay was performed in triplicate.

Anchorage-independent growth. Soft agar colonization assays were performed as described (33). Briefly, a bottom agar (Difco) of 0.7% in DMEM + 10% FBS was allowed to harden in all wells of a 24-well plate. Cells were suspended in a 0.3% agar/DMEM + 10% FBS at a concentration of 1 × 10³/mL. Cells were plated on top of the bottom agar in triplicate wells. Colony number was assessed after 14 days of growth at 37°C, 5% CO₂. All colonies that consisted of >50 cells were counted per well. Mean colony number was calculated and graphed in Microsoft Excel. Each assay was performed in triplicate.

Clonogenicity. Cells were seeded in triplicate wells of six-well plates at 1 × 10³ cells per well in DMEM + 10% FBS. Colonies were grown at 37°C, 5% CO₂ for 21 days. Cells were then fixed and stained with Giemsa. Stained cells were dried and all colony-forming units per well were counted. Mean colony numbers were calculated and graphed in Microsoft Excel. Each assay was performed in triplicate.

Spontaneous metastasis assay. Experiments were performed under an approved National Cancer Institute Animal Use Agreement. Six-week-old athymic NCr−nu/nu females were obtained from Charles River Laboratories (National Cancer Institute-Frederick Animal Production Area, Frederick, MD). Cells were labeled with the CellTracker Green CMFDA (Invitrogen) at a final concentration of 25 μmol/L in serum-free DMEM. Labeled cells were trypsinized, detached, and resuspended in 1 × HBSS to a concentration of 5 × 10³/mL. Using a 27-gauge × 1/2 inch hypodermic needle, a 0.1-mL volume of cells (5 × 10³) was delivered by lateral tail vein injection. Mice were euthanized at either 1, 6, or 24 h after injection. Five mice were injected per cell line per time point. On euthanization, lungs were dissected out for ex vivo imaging with a fluorescent inverted microscope. Images of 10 representative regions of each lung were captured using Openlab software and lung tissue was preserved in 10% formalin. The number of cells per image was counted using the Openlab software automatic feature counter and histograms were constructed in Microsoft Excel. Statistical significance was determined using one-way ANOVA and a Tukey honest significant difference (HSD) test and experiments were performed in duplicate.

Ex vivo microscopy. Experiments were performed under an approved National Cancer Institute Animal Use Agreement. Female athymic NCr−nu/nu mice of 6 weeks of age were purchased from Charles River Laboratories (National Cancer Institute-Frederick Animal Production Area, Frederick, MD). Cells were labeled with the CellTracker Green CMFDA (Invitrogen) at a final concentration of 25 μmol/L in serum-free DMEM. Labeled cells were trypsinized, detached, and resuspended in 1 × HBSS to a concentration of 5 × 10³/mL. Using a 27-gauge × 1/2 inch hypodermic needle, a 0.1-mL volume of cells (5 × 10³) was delivered by lateral tail vein injection. Mice were euthanized at either 1, 6, or 24 h after injection. Five mice were injected per cell line per time point. On euthanization, lungs were dissected out for ex vivo imaging with a fluorescent inverted microscope. Images of 10 representative regions of each lung were captured using Openlab software and lung tissue was preserved in 10% formalin. The number of cells per image was counted using the Openlab software automatic feature counter and histograms were constructed in Microsoft Excel.
Immunohistochemistry. Tumor tissue from 20 patients with breast cancer was collected by the Laboratory of Pathology, National Cancer Institute (Bethesda, MD) and anonymized. Tissue specimens were formalin fixed and paraffin embedded and 4-μm sections were mounted onto silanized glass slides. Histopathologic characteristics of a H&E-stained section were confirmed in consultation with a pathologist. Immunohistochemical staining was performed as described previously (33). The following primary antibodies were used: mouse monoclonal anti-Nm23-H1 (BD Biosciences PharMingen) and rabbit polyclonal anti-human EDG2 (Novus Biologicals). Stained sections were examined microscopically at ×400 magnification and scored from 0 to 3+ in consultation with a pathologist.

Results

EDG2 restores tumor cell motility, invasion, and adhesion to Nm23-H1–suppressed tumor cells. In an attempt to define the mechanism of action of Nm23-H1 metastasis suppression, we previously reported that (a) overexpression of Nm23-H1 significantly reduced the in vivo metastasis and in vitro motility of MDA-MB-435 tumor cells (3, 7, 20, 32), (b) overexpression of Nm23-H1 was associated with the reduced expression of the LPA receptor EDG2, and (c) transient reexpression of EDG2 in Nm23-H1–overexpressing cells (H1-177) significantly augmented their in vitro motility to either FBS or LPA (20). Although the enhancement of H1-177 motility by EDG2 reexpression was substantial (60-fold), it remained only 50% of control-transfected MDA-MB-435 cells (C-100). Two hypotheses were tested in the following set of experiments. First, reexpression of EDG2 in Nm23-H1–overexpressing H1-177 cells stimulated in vitro and in vivo aspects of tumor metastasis. Second, the phenotypic effects of EDG2 overexpression may be more pronounced on stable expression.

Clonal cell lines stably expressing Nm23-H1 and EDG2 were constructed. H1-177 cells, stable Nm23-H1 transfectants of MDA-MB-435 tumor cells, were transfected with an EDG2 expression construct or empty vector and clonally selected. Figure 1A shows an immunoblot of protein lysates from the transfectants. The Nm23-H1 transfectant of MDA-MB-435 cells, H1-177, expressed 5-fold increased Nm23-H1 and 5-fold decreased EDG2 compared with a control transfectant of the same line (C-100), in agreement with previously published data (20). Two stable clonal EDG2 transfectants of H1-177 (H1EDG2 c1-2) expressed 10- to 20-fold greater EDG2 than two vector transfectants of the same line (H1vector c1-2). The band for EDG2 is broad because this G protein–coupled receptor is heavily glycosylated. All of the H1-177 transfectants exhibited comparably high levels of Nm23-H1.

The activity of these cell lines was assessed in multiple in vitro assays for aspects of tumor metastasis. Boyden chambers were

![Figure 1](image_url)
used to assay motility to either FBS or LPA (Fig. 1B; Supplementary Fig. S1). Cells with high Nm23-H1 levels and low EDG2 levels, H1-177 and H1vector clones, exhibited almost no motility. Metastatic C-100 cells, which express low Nm23-H1 levels and high EDG2 levels, were highly motile. Forced stable expression of EDG2 in the H1-177 background significantly increased the motility of H1-177 cells (H1vector versus H1EDG2, *P* < 10⁻¹¹, *t* test) and completely restored motility to the level observed for C-100 and even elevated motility by 1.3- to 1.9-fold (H1EDG2 versus C-100, *P* < 0.06, *t* test). This enhancement is perhaps reflective of higher levels of EDG2 expression (Fig. 1A).

A component of cell motility and invasion is the capacity of the tumor cell to form focal adhesions at the leading edge of the cell. Nm23-H1 has been shown to inhibit cell adhesion to both endothelial cells and extracellular matrix components (8, 9). In addition, LPA signaling has been reported to be important for the localization and activation of the focal adhesion kinase FAK (34, 35). Given this information, the adhesive properties of the Nm23-H1–expressing and EDG2-expressing cell lines were assayed. Cells were seeded in wells coated with fibronectin and allowed to adhere over a 30-min time frame. The number of adherent cells retained after washing was estimated by crystal violet staining. Compared with C-100 cells, Nm23-H1–overexpressing H1-177 inhibited adhesion by ~50% (C-100 versus H1-177; *P* = 1.6 × 10⁻⁵, *t* test; Fig. 1D). Forced expression of EDG2 restored adhesion to H1-177 cells (H1vector versus H1EDG2; *P* = 3 × 10⁻⁵, *t* test). Taken together, these data indicate that Nm23-H1 suppression of cell adhesion, motility, and invasion to FBS or LPA can be explained by diminished expression of the LPA receptor EDG2. Stable as opposed to transient transfection of EDG2 exerted a more pronounced effect on motility in vitro. Suppression of any or all of these phenotypes may contribute to overall metastasis suppression.

Figure 2. Nm23-H1 suppresses cell survival in the murine lung and coexpression of EDG2 rescues this phenotype. A, MDA-MB-435, C-100, and H1-177 cells were fluorescently labeled with CMFDA and injected via lateral tail vein into athymic mice. The number of cells in the lungs was quantified at 1, 6, and 24 h after injection and the mean number for each cell line and time point is represented by histogram. B, same experiment as represented in A with H1vector and H1EDG2 clones. C, representative micrographs of regions of the lung at 1 and 6 h after injection for the C-100, H1-177, an H1vector, and an H1EDG2 cell line at ×100 magnification.
Tumor cell retention in the lungs is enhanced by EDG2 expression. Tumor cell adherence, motility, and invasion are important facets of the initial invasion process but may also be involved in adherence, arrest, extravasation, and survival at a distant site, later steps in metastasis. The capacity of Nm23-H1 to suppress, and the capacity of EDG2 to restore tumor cell retention in the lungs, was assessed. The C-100, H1-177, and H1-177–derived clonal transfectants were labeled with the green fluorescent dye CMFDA to track cells in vivo. Equivalent numbers of cells were injected via the lateral tail vein into female athymic mice. Tail vein injection selects for tumor cell deposition to the lung and MDA-MB-435–derived cells almost exclusively metastasize to the murine lungs; thus cell arrest, extravasation, and early cell survival in the lung were assessed (Fig. 2). The lungs of five anesthetized mice per cell line were dissected and examined by fluorescent microscopy at 1, 6, and 24 h after injection. Equivalent numbers of cells for all lines were evident in the lungs at 1 h after injection as depicted in Fig. 2A to C. However, at 6 h after injection, distinct differences in the number of retained cells emerged between the transfectants. Figure 2A graphically shows that fewer Nm23-H1–expressing H1-177 cells were retained in the lungs at this time point compared with C-100 control transfectants (1.5% versus 9.7% of mean cell numbers; \( P < 0.05 \), ANOVA and Tukey HSD test). At 24 h, the number of tumor cells was below the sensitivity of the assay. Photomicrographs of the C-100 and H1-177 cells at the 1- and 6-h time points are shown in Fig. 2C. The relative advantage of the C-100 cell line after injection into the circulation may partially explain its enhanced metastatic propensity compared with H1-177 cells if the cells survive and propagate.

Figure 2B shows a similar experiment to test the hypothesis that EDG2 restores the capacity of H1-177 cells to arrest, extravasate, and survive in the lung. A H1-177 vector-transfected clone with low EDG2 expression exhibited only 5% cell retention rate at 6 h, whereas H1EDG2 clones exhibited a 40% to 70% cell retention rate (\( P < 0.01 \), ANOVA and Tukey HSD test). EDG2 therefore conferred
an in vivo advantage to H1-177 nonmetastatic cells that may contribute to an enhanced metastatic capacity.

EDG2 expression does not affect growth and clonogenicity of Nm23-H1–suppressed tumor cells. EDG2 has been implicated in the promotion of tumor cell growth as well as motility (28). In addition, Nm23-H1 inhibited anchorage-independent growth in soft agar (7). To determine if reduced EDG2 expression was also involved in growth inhibition in the present model system, anchorage-dependent and anchorage-independent growth was assessed in the C-100, H1-177, and H1-177–derived clonal transfectants. No difference was observed between the anchorage-dependent growth rate of the C-100 and H1-177 cells over a 96-h time course using a MTT assay (Fig. 3A). Stable expression of EDG2 in H1-177–derived cells did not affect the growth rate of these cells. In clonogenicity assays, C-100 and H1-177 cells are equally clonogenic (data not shown). The H1-177–derived lines, H1vector and H1EDG2 clones, were all ~2-fold more clonogenic than both C-100 and H1-177, but no significant difference in clonogenicity was observed between the H1vector and H1EDG2 lines (Fig. 3B). However, the colony morphology differed between control and EDG2-expressing clones (Fig. 3C). Both the H1-177 line and the vector-transfected derivatives of H1-177 formed tighter, more compact colonies, whereas colonies formed by high EDG2-expressing cells (C-100 and H1EDG2 clones) were more dispersed, perhaps reflective of their motile phenotype.

Anchorage-independent growth in soft agar was also assessed. Nm23-H1–expressing H1-177 cells have a diminished capacity to form colonies in soft agar compared with metastatic C-100 cells in serum-rich (10% FBS) conditions (7). Restoration of EDG2 expression in the H1-177 background did not significantly enhance anchorage-independent colonization when compared with vector transfectants under the same serum conditions (Fig. 3D).

EDG2 restores in vivo metastatic propensity to Nm23-H1–suppressed tumor cells. Given that EDG2 can overcome some metastatic correlates known to be suppressed by Nm23-H1, we hypothesized that EDG2 can overcome Nm23-H1–mediated in vivo metastasis suppression. To assess the effect of EDG2 on Nm23-H1–suppressed tumor cells for the entire metastatic process,
spontaneous metastasis was assayed with the H1vector and H1EDG2 cell lines. Metastatic C-100 cells, H1vector, and H1EDG2 were injected into the mammary fat pads of female athymic mice. Primary tumors became visible 30 days after injection and were measured weekly for 3 months. Figure 4A summarizes tumor volume over time for all five cell lines. No difference in primary tumor volume and growth rate was apparent in agreement with in vitro proliferation and colonization data.

EDG2 is critical for invasive growth in vitro (Fig. 1); therefore, the cell lines might exhibit differences in local invasion from the primary tumor in vivo. Primary tumor sections were stained with H&E and representative images of the tumor borders are shown in Fig. 4B. Whereas the border between the tumor and the mammary fat pad was distinct for the H1vector tumor cells, the C-100 and H1EDG2 tumor cells were more invasive into the surrounding tissue.

Table 1 lists the pulmonary metastatic abilities of the transfectants 4 months after injection based on examination of H&E-stained step sections through the lungs. As a positive control, the C-100 line produced pulmonary metastases in 83.3% to 93.8% of mice over two independent experiments. Exogenous expression of Nm23-H1 in MDA-MB-435 cells has previously been shown to reduce the incidence of pulmonary metastases by ~90% (7). Here, we observed that two independent H1vector tumors produced pulmonary metastases in 51.9% of mice, approximately a 40% reduction in metastatic incidence compared with C-100 cells. This overall higher incidence of metastasis may have resulted from an additional round of transfection and clonal selection. Two independent H1EDG2 lines produced pulmonary metastases in 90.4% of mice, an incidence significantly higher than that observed for H1vector lines (P = 2.4 × 10^{-5}, Fisher’s exact test) but not statistically different from C-100 (P = 1.00, Fisher’s exact test). The number of pulmonary metastases derived from the H1EDG2 lines was also significantly higher than that observed for the H1vector lines when data from both experiments were pooled (P = 0.0035, Fisher’s exact test). After determining that the number of metastases in C-100 could not be pooled across experiments, comparison of the number of pulmonary metastases between the H1EDG2 lines and the C-100 positive control revealed that the number of metastases derived from C-100 is higher than the H1EDG2 lines in the first experiment (P = 0.01, Fisher’s exact test) but not in the second experiment (P = 0.67, Fisher’s exact test). These data establish that EDG2 expression overcomes Nm23-H1 inhibition of in vivo metastasis, in terms of incidence data usually reported in spontaneous metastasis assays. The number of metastases per mouse was also nearly restored to the levels exhibited by C-100 cells.

Nm23-H1 expression inversely correlates with EDG2 expression in a human breast tumor cohort. Given that EDG2 expression significantly augmented the incidence of pulmonary metastases in the Nm23-H1–suppressed MDA-MB-435 model, we addressed whether the inverse correlation of Nm23-H1 and EDG2 expression was generalizable to clinical breast cancer. In a previous report, a significant inverse relationship was identified between Nm23-H1 and EDG2 expression in a microarray data set of breast tumor cohort (20). This analysis was limited in that only relative transcript levels were measured and mRNA was collected from bulk tumor tissue, which also contained stromal tissue. To validate the inverse relationship in clinical samples, the relative protein expression of Nm23-H1 and EDG2 in tumor cells was analyzed by immunohistochemistry using a cohort of 18 breast tumor specimens. Serial sections were stained for Nm23-H1 and EDG2 by standard immunohistochemical procedures. Expression of Nm23-H1 and EDG2 was scored on a standard intensity scale of 0 to 3+ in each tumor region in consultation with a pathologist. These data are summarized in Supplementary Table S1 and selected photomicrographs are shown on Fig. 5. An inverse correlation between Nm23-H1 and EDG2 protein was observed in most of these clinical breast tumor samples (r = −0.73; P = 0.004, Pearson correlation). Tumor regions with low Nm23-H1 expression had high levels of EDG2 in 3 of 18 samples (Fig. 5, tumors 1–3) and the inverse relationship was observed in 14 of 18 samples (Fig. 5, tumors 12, 16, and 17). Heterogeneous expression of both Nm23-H1 and EDG2 was observed in 1 of 18 tumors (Fig. 5, tumor 8). The data confirm an inverse correlation of tumor Nm23-H1 and EDG2 expression in a breast tumor cohort.

### Table 1. In vivo metastatic potential of transfected, clonal MDA-MB-435 cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Percentage (incidence) with pulmonary metastases</th>
<th>Median (range) pulmonary metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td>C-100 (positive control)</td>
<td>93.8 (15/16)</td>
<td>83.3 (10/12)</td>
</tr>
<tr>
<td>H1vector c1</td>
<td>58.3 (7/12)</td>
<td>57.1 (8/14)</td>
</tr>
<tr>
<td>H1vector c2</td>
<td>42.9 (6/14)</td>
<td>50 (6/12)</td>
</tr>
<tr>
<td>H1EDG2 c1</td>
<td>81.3 (13/16)</td>
<td>91.7 (11/12)</td>
</tr>
<tr>
<td>H1EDG2 c2</td>
<td>100 (12/12)</td>
<td>91.7 (11/12)</td>
</tr>
</tbody>
</table>

NOTE: To determine the suitability of pooling results between clonal cell lines and across experiments, incidence and numbers of metastases were compared using Fisher’s exact test and the Wilcoxon rank sum test, respectively. The number of metastases for experiments 1 and 2 for the C-100 cell line was not statistically different but trended toward being distinct (P = 0.10); thus, the data for number of metastases in both experiments for the C-100 cell line were not pooled. Statistical significance of the incidence and number of metastases between groups based on pooled data was determined using a Fisher’s exact test and Wilcoxon rank sum test, respectively.

*P = 2.4 × 10^{-5}.
† P = 0.0035.
Discussion

Nm23-H1 was first shown to have metastasis suppressive capacity nearly two decades ago, and despite intense research, a mechanism by which it impinges on the metastatic process has not been confirmed. Based on abrogation of Nm23-H1 motility suppression in vitro, two potential mechanisms of metastasis suppression have been posited. First, mutational analysis revealed that Nm23-H1 motility suppressive effects correlate with its histidine kinase function toward substrates such as KSR, succinic thiokinase, and ATP citrate lyase (30, 36, 37). Second, interactions with c-PRUNE, EBV proteins, and other binding proteins are thought to bind and sequester bioavailable Nm23-H1, thereby antagonizing the motility suppressive functions of Nm23-H1 (38–40).

We present data herein identifying a third potent contributor to Nm23-H1 metastasis suppression: transcriptional down-regulation of the LPA receptor EDG2. In a previous study, we identified differential gene expression between Nm23-H1–transfected MDA-MB-435 cells and transfectants expressing only a control vector or two site-directed mutants of Nm23-H1 incapable of suppressing tumor cell motility in vitro (20). Although nine genes, including genes for cell surface receptors, adhesion molecules, and secreted growth factors, were exhaustively validated as being inversely proportional to Nm23-H1 expression, only one, EDG2, reestablished a motile phenotype when expressed in H1-177 cells (20). Here, these data were extended to show that EDG2 also restores metastasis. The incidence of pulmonary metastases increased from 51.9% in Nm23-H1–overexpressing H1-177 vector transfectants to 90.4% in cells coexpressing EDG2, comparable with that of vector-transfected MDA-MB-435 cells, perhaps creating localized gradients of LPA to prompt invasion. It has long been hypothesized that metastatic tumor cells must adapt to the new microenvironment of the metastatic site (lungs, liver, bone, etc.) to colonize. It is tempting to postulate that, in addition to tissue-specific microenvironmental interactions, tumor cells also coopt widely available factors, such as LPA or its phospholipid precursors, to facilitate their survival.

A role for EDG2 in prompting metastasis has already been established, but the present article links Nm23-H1 function to EDG2 expression. The role of EDG2 in overcoming Nm23-H1 suppression of metastasis seems to involve several functions. This LPA receptor completely restores a motile, invasive, and adhesive phenotype to Nm23-H1–suppressed cancer cells in in vitro assays. In vivo, these characteristics contribute to enhanced local invasion from the primary tumor and an increase in tumor cell retention in the murine lung. This enhanced retention could be attributed to increased efficiency of tumor cell adhesion, arrest, extravasation, and/or survival at the distant site. Further study of EDG2 signaling in metastatic cells is required to determine if all of these in vitro and in vivo effects are mediated by identical or distinct pathways and which pathway(s) is important for prompting metastasis. LPA receptor signaling is known to stimulate focal adhesion complex components, such as FAK and Src, known regulators of integrin-mediated invasion and facilitators of anoikis resistance (41, 42). In particular, in an orthotopic model, LPA-mediated metastasis was inhibited by LY294002, an inhibitor of the Src substrate phosphatidylinositol 3-kinase (43).

LPA is an abundant, fairly ubiquitous phospholipid, which is a major constituent of blood serum, found at levels ranging from 25.9 to 98.3 μmol/L (44). Roughly half of serum LPA is derived from the generation of phospholipids from activated platelets and the subsequent conversion to LPA by autotaxin (45). Blood levels of the phospholipase autotaxin are relatively constant; however, several tumor cells produce and secrete this enzyme, including MDA-MB-435 cells, perhaps creating localized gradients of LPA to prompt invasion. An inverse correlation between EDG2 and Nm23-H1 expression was established in a cohort of human breast carcinomas, suggesting that the relationship between these two proteins is of clinical relevance. Taken together with our genetic analysis of the antagonistic relationship between these proteins in a spontaneous metastasis assay, these data support the hypothesis that EDG2 inhibitors will be advantageous for the prevention of metastatic breast colonization. Given that EDG2 seems to promote metastasis by improving the efficiency of early steps in metastasis, invasion, intravasation, and extravasation, inhibitors would presumably provide the most benefit in the adjuvant setting for low Nm23-H1–expressing tumors.

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Figure 5. Nm23-H1 and EDG2 expression is inversely related in a breast tumor cohort. Micrographs of immunohistochemical staining for Nm23-H1 and EDG2 protein in seven human breast tumors. Magnification, ×400.


Nm23-H1 Suppresses Metastasis by Inhibiting Expression of the Lysophosphatidic Acid Receptor \textit{EDG2}

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