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Characterizing the expression and function of metastasis suppressor protein Nm23-H1 in triple-negative breast cancer

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Abstract

Characterizing the expression and function of metastasis suppressor protein Nm23-H1 in triple-negative breast cancer

By Rachel Tobin

Triple negative breast cancers (TNBCs) are a heterogeneous group of breast cancers characterized by poor prognoses due to the lack of three receptors: estrogen receptor (ER), progesterone receptor (PR), and HER2/neu. As a result, treatments for TNBCs are limited to conventional chemotherapy, and diagnosis is correlated with highly metastatic, recurrent, and incurable disease. The metastasis suppressor protein Nm23-H1 is thought to inhibit metastasis by controlling cell motility, invasion, cell-cell adhesion, and cytoskeletal reorganization. We examined the roles of Nm23-H1 in human TNBC cell lines and its effects on downstream Rho GTPase signaling, cell migration, and cell invasion. Nm23-H1 protein is expressed in morphologically distinct TNBC cell lines. Nm23-H1 was silenced in the epithelial HCC1806 and mesenchymal BT549 cell lines using transient siRNA techniques to determine the functional roles of Nm23-H1 in TNBCs. These cells were also treated with megestrol acetate (MA), a drug that increases Nm23-H1 protein levels, to determine the effects of increased Nm23-H1 in TNBC cells. Interestingly, Nm23-H1 appeared to display tissue-specific functional roles in epithelial versus mesenchymal cells. In the epithelial HCC1806 cell line, Nm23-H1 acts in its defined role as a metastasis suppressor. Paradoxically, our data suggests that Nm23-H1 appears to function as an oncogene in the mesenchymal BT549 cell line. Therefore, Nm23-H1 could serve as a potential therapeutic target in epithelial but not mesenchymal TNBC phenotypes, since Nm23-H1 exerts oncogenic effects in mesenchymal tissue. Our data support the notion that targeting
Nm23-H1 in epithelial TNBC tumors could serve as a novel therapeutic strategy to reduce the high mortality rates associated with TNBC metastasis.
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CHAPTER 1: Introduction

Breast Cancer Statistics

In the United States in 2015 alone, there are expected to be over 200,000 newly diagnosed cases of invasive breast cancer and over 40,000 deaths as a result of the disease. Excluding cancers of the skin, breast cancer is the most frequently diagnosed cancer in women. Invasive breast cancer is also the second leading cause of cancer death in women, following lung cancer. For all breast cancer stages combined, the 5-, 10-, and 15-year survival rates are 89%, 83%, and 78% respectively (1). However, disparities exist in clinical outcomes and survival due to race. For all stages combined, the 5-year survival rate for Caucasian women is 90%, whereas the 5-year survival rate for African American women is 79% (1). Additionally, for Caucasian women in the United States, there are approximately 28.3 deaths per 100,000 cases of breast cancer, whereas for African American women, there are approximately 36.4 deaths per 100,000 cases (2). Studies have shown that in the United States, African American women have a 41% higher incidence of breast cancer related death than do Caucasian women (3).

Breast Cancer Subtypes

Breast cancer is not thought to be one disease, but instead a group of cancers with different responses to therapy and prognoses. Historically, breast cancers have been classified according to patient age, axillary lymph node involvement, hormone receptor status, human epidermal growth factor receptor 2 (HER2) status, and histological features in order to determine patient prognosis and therapy. The main hormone receptors used for classification are estrogen receptor (ER), progesterone receptor (PR), and HER2. This classification system has been effective for predicting prognosis in groups of
patients; however, this system has not been accurate in assessing risks for individuals. Patients with cancers of similar features according to this system have had very different clinical outcomes (4).

Novel advances in molecular techniques, in particular gene expression profiling, have been used to create a more refined classification system for various breast cancers. Over the past ten years, four main subtypes of breast cancer, known as the four molecular “intrinsic” subtypes have been identified as follows: Luminal A, Luminal B, HER2-enriched, and basal-like. A normal breast-like group has also been identified (5). More recently, a novel intrinsic subtype has been discovered, which is known as Claudin-low (6).

The luminal A subtype comprises about 40% of all breast cancers. These tumors are characterized as ER+ or PR+, HER2- and are associated with the most favorable short-term prognosis as they are generally more slowly growing and less aggressive than other subtypes. Due to the expression of hormone receptors, hormonal therapy (e.g. tamoxifen) is used as an effective treatment option. The luminal B subtype, which encompasses about 10-20% of breast cancers, expresses hormone receptors (ER+ or PR+), similar to the luminal A subtype. However, these tumors also overexpress HER2 and have high rates of proliferation. Treatment options for these tumors include trastuzumab (Herceptin), which inhibits HER2 signaling. About 10% of all tumors are categorized as HER2 enriched. HER2 is a growth-promoting protein and its overexpression results in more aggressive growth and poorer short-term prognosis. HER2 targeted therapies like trastuzumab have improved prognosis. The basal-like subtype encompasses about 10-20% of all breast tumors and is characterized as ER-, PR-,
and HER2-. Due to the lack of these three receptors, many cancers of the basal-like subset can also be classified as triple-negative. Triple-negative breast cancer is most common in premenopausal women, African American women, and patients with a \textit{BRCA1} gene mutation. There are currently no targeted therapies for this subtype (7). The recently discovered Claudin-low subtype is also triple-negative (ER-, PR-, HER2-), possesses unique characteristics similar to mammary stem cells, and is associated with poor prognoses (6).

\textbf{Triple Negative Breast Cancer}

Triple-negative breast cancers (TNBCs) are a heterogeneous class of breast cancers characterized by a lack of three receptors: ER, PR, and HER2 overexpression. Due to a lack of these receptors, TNBCs lack molecular targets, and therefore treatments are limited to conventional chemotherapy. Current chemotherapeutic treatments for TNBCs include taxanes, platinum agents, anthracyclines, and biologic agents (8). Also, because of the lack of targeted therapies, diagnosis is correlated with highly metastatic, recurrent, and incurable disease (9). TNBC tumors are generally larger at diagnosis, have lymph node involvement, and are more aggressive. TNBCs comprise about 10-20% of all breast cancers and tend to disproportionally affect premenopausal women, African-American, and Hispanic women. TNBCs are likely to arise in patients with a mutation in the \textit{BRCA1} gene, which is involved in repairing damaged DNA (10).

In 2010, Perou et. al. demonstrated the heterogeneity found in the triple-negative subtype by applying molecular classification to 470 different breast tumors (11). They discovered that the majority of tumors that were classified as triple-negative were of the basal-like phenotype (about 50-75%), however, all other subtypes were present, which
includes the luminal A, luminal B, HER2 enriched, and claudin-low groups. The basal-like phenotype is characterized by the unique expression of cytokeratins 5, 6, or 17, and the vast majority of these cancers have a mutated form of the tumor suppressors \( p53 \) and \( RB \), resulting in increased proliferation (11).

In order to further identify molecular subtypes of TNBCs to understand potential targeted treatments, Lehmann et. al. analyzed gene expression profiles from 21 breast cancer data sets, which included 587 TNBC tumors and 25 different TNBC cell lines (10). They identified six TNBC subtypes: basal-like 1 (BL1), basal-like 2 (BL-2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR). The BL1 subtype is enriched in the expression of genes that are associated with cell cycle progression and proliferation. The BL-2 subtype displays unique gene expression patterns of growth factor signaling, gluconeogenesis, and glycolysis. The IM subtype expresses genes related to immune processes. The M subtype displays gene ontologies related to cell motility, and the MSL subtype is similar to the M subtype, however the MSL subtype also expresses genes related to growth-factor signaling and resembles mammary stem cells. Finally, the LAR subtype is enriched in androgen responsive hormonal pathways, despite being ER- (10). In this study, the cell lines HCC1806 (BL-2 subtype) and BT549 (M subtype) were used to examine TNBC cells \textit{in vitro}.

\textbf{Metastasis Dangers and Overview of Mechanism}

Metastasis is the process by which tumor cells leave the primary site and form secondary tumors at distant sites in the body. Metastasis is responsible for about 90% of cancer deaths (12). Due to the aggressive nature of TNBCs, these cancers result in a
A disproportionate number of metastatic cases and deaths (13). TNBC relapse and metastasis tends to occur preferentially in the visceral organs, as opposed to the bone, which is often seen in breast cancers expressing hormone receptors. The lung and brain are also common sites for secondary tumor formation in TNBCs (14).

Metastasis is an inefficient and complex process, as millions of cells may be released by the tumor each day, yet only a few are able to colonize at distant organs. In order for a tumor cell to successfully metastasize, it must lose adhesion to neighboring cells, enter and survive in circulation, exit circulation into a novel tissue, and colonize the distant site. This task is no easy feat, as the cancer cell must survive an array of host defenses, including physiological barriers, sheering forces, and the immune system (15).

Metastasis occurs in a set of discrete steps, known as the “metastatic cascade”. Metastases begin from primary, epithelial, neoplastic lesions. Tumor cells are released from this primary site, which must then breach the basement membrane in order to enter into circulation. In order to do so, cells must undergo epithelial-mesenchymal transition (EMT), in which polarized epithelial cells acquire characteristics of mesenchymal stem cells, allowing invasion into tissues. Protein-degrading enzymes, known as matrix metalloproteinases (MMPs), are released to cleave components of the extracellular matrix. The next barrier that cancer cells must overcome is anoikis, a type of cell death, which is induced by loss of cell adhesion. Anoikis suppression is needed in order for cells to colonize distant sites. If this is successful, tumor cells then need to gain access to the blood vessels for transport throughout the circulation. Next, tumor cells intravasate into the blood vessels, are transported throughout the circulation, and then extravasate into a secondary site. The “seed and soil hypothesis” suggests that the secondary site that
the tumor cells seed is not random, but instead tumor cells can only colonize a secondary site in which the microenvironment (soil) is suitable for growth. It has recently been proposed that tumor cells may create a “pre-metastatic niche” at the target site before the process of metastasis begins (16).

*The Epithelial-Mesenchymal Transition (EMT): An Important Step in the Metastatic Cascade*

Epithelial-mesenchymal transition (EMT) is a process in which polarized epithelial cells lose their adhesive properties and become mesenchymal stem cells, which possess migratory and invasive properties. The mesenchymal stem cell phenotype is characterized by increased invasiveness, migratory capacity, production of ECM components, and resistance to apoptosis (17).

EMT is not a process unique to cancer progression, but is also a part of normal development. EMT, which occurs during embryo formation and organ development in order to generate a multitude of cell types, is known as type 1 EMT. Type 2 EMT is associated with tissue regeneration and organ fibrosis in response to inflammatory signals. Type 3 EMT, which is most relevant to this study, is EMT that is associated with cancer growth and metastasis (17). Cancer cells undergoing EMT possess the potential to break through the basement membrane and travel throughout the body due to cytoskeletal rearrangement (18). This process is reversible and the mesenchymal phenotype is transient, as cancer cells must undergo a mesenchymal-epithelial transition (MET) in order to successfully form a secondary tumor site. Studies have shown that breast cancer metastases are epithelial, rather than mesenchymal, which demonstrates the plastic nature of tumor cells (19).
Several proteins have been identified that serve as markers for EMT. Zinc finger E-box binding homeobox 1 (Zeb1) has been identified as repressing transcription of E-cadherin, which is important for cell-cell adhesion (20). The Snail and Twist families are considered the master regulators of EMT, modulating cell adhesion and cytoskeletal changes (21). Understanding the mechanisms of this transition during cancer progression could prove to be insightful for the inhibition of metastasis and to reduce mortalities associated with TNBCs.

**Metastasis Suppressors and Nm23-H1**

Metastasis suppressor genes (MSGs) are able to suppress the development of metastases, the most deadly aspect of cancer, without affecting the growth of the primary tumor. Over twenty MSGs have been identified *in vivo* (22). Metastasis suppressor genes are a class of genes distinct from oncogenes, which promote carcinogenesis, and tumor suppressor genes, which suppress growth of the primary tumor (23).

In 1988, Non-metastatic gene 23-H1 (Nm23-H1) or Nme1 was the first MSG identified due to its reduced expression in a highly metastatic murine melanoma cell line (K-1735 TK). Clinically, decreased expression of Nm23-H1 is associated with poor prognosis in melanoma, breast, colon, and stomach cancers (24). The gene is located on chromosome 17q21 and codes for a 166 amino acid protein, which is 18.5 kDa in size. Nm23-H1 is a highly conserved gene that gives rise to the nucleoside diphosphate kinase A (NDKA) protein product; it functions as a nucleoside diphosphate kinase (NDPK), which transfers a phosphate from a nucleotide triphosphate to a nucleotide diphosphate. In addition to Nm23-H1’s NDPK activity, it also possesses serine autophosphorylation activity. The Nm23-H1 family consists of over eight isoforms, which are involved in
cellular adhesion, migration, differentiation, signal transduction, and apoptosis. Nm23-H1 is the isoform that encodes the alpha subunit of NDPK and is most correlated to a metastasis suppressor function. Although the mechanism of how it suppresses metastasis remains unclear, it is thought to function as a GTPase activating protein and regulator of the Rho family GTPases (22).

**The Rho GTPases and Their Oncogenic Link**

The Rho GTPases are a family of small G proteins, which regulate cell migration, the cell cycle, cell polarity, and the cytoskeleton. These proteins function as “switches”, inactive when in a GDP-bound form, but active when GDP is swapped for GTP. This exchange of GDP for GTP is catalyzed by guanine nucleotide exchange factors (GEFs), and the “switch” is turned off when GTPase activating enzymes (GAPs) catalyze intrinsic GTPase function (25).

This study focuses on three specific members of the Rho GTPase family: RhoA, Rac1, and Cdc42. These GTPases are all ubiquitously expressed and known to promote cell cycle progression, mesenchymal cell migration, cell survival, and angiogenesis. Many tumors have shown increased gene expression of these Rho GTPases. In breast cancer specifically, increased gene expression of Rac1, Cdc42, and RhoA have been shown to increase progression and metastasis, yet the specific mechanisms of action in TNBCs remain unclear (26).

**Hypothesis, Specific Aims, and Strategy**

**Hypothesis:** Given its defined role as a metastasis suppressor protein, we hypothesize that Nm23-H1 could serve as a possible therapeutic target for reducing the metastatic potential of TNBCs. We propose that, by increasing levels of Nm23-H1, TNBC cells
could be confined to the primary tumor site, thereby reducing or preventing metastatic TNBC. We have developed the following specific aims to test our hypothesis:

**Specific Aim 1**: Characterize Nm23-H1 expression in epithelial versus mesenchymal triple-negative breast cancer.

**Specific Aim 2**: Examine the functional role of Nm23-H1 in epithelial and mesenchymal triple-negative breast cancer cell lines using siRNA silencing.

**Specific Aim 3**: Investigate the functional effects of increasing Nm23-H1 expression using megestrol acetate (MA).

**Strategy**: We employed siRNA-silencing techniques to knock down Nm23-H1 protein expression in order to determine whether Nm23-H1 serves as a metastasis suppressor using non-metastatic (HCC1806) and metastatic (BT549) TNBC cells. We also examined the effect of Nm23-H1 silencing on the expression levels of the small Rho GTPases (downstream targets of Nm23-H1), cellular migration, and invasion in both TNBC cell lines. Additionally, we examined the effects of increasing levels of Nm23-H1 in TNBC cells by using megestrol acetate (MA), a drug that increases gene expression of Nm23-H1. These experiments were performed in an epithelial and a mesenchymal cell line to investigate the differences between these two TNBC phenotypes, which could provide insight into the relationship between Nm23-H1, the Rho GTPases, and EMT. Further characterizing EMT, an important step in the metastatic cascade, could prove valuable for understanding the aggressive behaviors of TNBCs and thereby reducing high mortality rates associated with TNBC metastasis.
CHAPTER 2: Nm23-H1 Expression in Triple-Negative Breast Cancer Cell Lines

Specific Aim 1: Characterize Nm23-H1 expression in epithelial versus mesenchymal triple-negative breast cancer.

Rationale: Nm23-H1 has been shown to suppress metastasis in a wide variety of cancers, such as melanoma, gastric, ovarian, cervical, and hepatocellular cancers. Previous studies suggest that Nm23-H1 prevents metastasis in breast cancers, but its link to TNBC specifically has not yet been investigated (27). Therefore, we wanted to investigate expression of Nm23-H1 in TNBC cell lines. By examining expression levels of Nm23-H1 in TNBC cell lines, we were able to investigate whether Nm23-H1 plays a role in this type of cancer.

Methods:

Pathway Studio: Pathway Studio is a software program that includes over 100,000 molecular networks that have been assembled from PubMed using MedScan (28). MedScan is a natural language processing algorithm that produces an output of structures that represent scientific relations, which include protein binding, promoter binding, protein modification, expression control, molecular transport, and molecular synthesis (29). Pathway Studio was used to analyze interactions of Nm23-H1 that exist in published scientific literature with RhoA, Rac1, and Cdc42.

Cell lines, Antibodies, and Reagents: RIPA cell lysis buffer was from Cell Signaling Technology, Inc. (Beverly, MA, USA), and protease inhibitor cocktail and phosphatase inhibitors were purchased from Sigma (Saint Louis, MO, USA). MDA-MB-231, HCC1806, and BT549 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were routinely maintained in Dulbecco’s Modification of
Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Clontech), 1 mM penicillin/streptomycin (Hyclone) and 2 µM L-glutamine (Invitrogen, Carlsbad, CA, USA).

**Western Blotting:** Total cell lysates were prepared using previously described methods (30). The primary antibody against Nm23-H1 was from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against β-actin (Sigma-Aldrich; Saint Louis, MO, USA), pFAK (Tyr397), total FAK, RhoA, Rac-1, and Cdc42 (BD Biosciences) were used for Western blots according to the manufacturer’s protocol. Bound primary antibodies were detected with anti-mouse or anti-rabbit peroxidase-coupled secondary antibodies (Southern BioTech; Birmingham, AL, USA) and developed by enhanced chemiluminescence (Luminata Classico Western HRP substrate; EMD Millipore Corp.; Billerica, MA, USA).

**Results:**

Using Pathway Studio, we determined that links between Nm23-H1 (NME1) and some of the small Rho GTPases exist in published scientific literature (Figure 1A). Using this software, we found that previous data has shown that Nm23-H1 inhibits Rac1, whereas Nm23-H1 induces expression of Cdc42. No links between Nm23-H1 and RhoA were identified using the Pathway Studio program. While generating this data, we decided to include IQGAP2 in our search. IQGAP2 is a GTPase activating protein that we thought may be of interest because it has been shown to be a downstream effector of Nm23-H1 and to interact with Rac1 and Cdc42 in melanoma cell lines. However, we did not find IQGAP2 to be of relevance in TNBC or to interact with Nm23-H1, so we did not include it in this project (31).
To characterize Nm23-H1 expression in TNBC, we examined basal protein levels in an epithelial cell line, HCC1806, and two mesenchymal cell lines, MDA-MB-231 and BT549 (Figure 1B). We found that all three cell lines endogenously expressed Nm23-H1, with the mesenchymal cell lines expressing the highest protein levels of Nm23-H1. We also examined basal protein levels of downstream targets of Nm23-H1: Rho, Rac1, and Cdc42 (Figure 4A). All three cell lines expressed these small Rho GTPases. Protein levels of focal adhesion kinase (FAK), a motility marker, were also examined. Expression of the phosphorylated form (pFAK) was found in the mesenchymal cell lines, MDA-MB-231 and BT549. However, the epithelial cell line, HCC1806, did not show pFAK expression. All three cell lines showed expression of total FAK (Figure 4A). β-actin was used as a loading control.
CHAPTER 3: Effects of Stable Knockdown of Nm23-H1 on Triple-negative Breast Cancer Cell Migration and Invasion

Specific Aim 2: Examine the functional role of Nm23-H1 in epithelial and mesenchymal triple-negative breast cancer cell lines using siRNA silencing.

Rationale: After showing that Nm23-H1 was differentially expressed epithelial and mesenchymal TNBC cell lines, we wanted to examine the functional effects of inhibiting the expression of the protein using siRNA silencing. By comparing Nm23-H1 control and knockdown TNBC cells, we can examine the migratory and invasive consequences of silencing Nm23-H1.

Methods:

siRNA silencing: Nme1-specific siRNA (SASI_Hs01-00050684; Sigma-Aldrich) was used to silence Nm23-H1 protein expression and a scrambled sequence siRNA (SIC001; Sigma-Aldrich) was used as a negative control. Transit-TKO transfection reagent (Mirus Bio LLC, Madison, Wisconsin, USA) was used to optimize siRNA transfection according to the manufacturer’s instructions. Briefly, Transit-TKO and Nme-1 or control siRNA were diluted separately in serum-free Opti-MEM media (Gibco) and incubated for 5 min at room temperature. The two solutions were gently mixed and incubated together for 30 min at room temperature. After incubation, the complex was added to the plated cells in complete culture media. Forty-eight hours post-transfection, cells were further assayed in Western blots, immunofluorescence staining, wound healing, spheroid migration, and Matrigel invasion assays as detailed below.

Western Blots: Following siRNA silencing, Western blotting was performed as described in Chapter 2 (Specific Aim 1).
**Immunocytochemistry:** HCC1806 and BT549 cells were grown on glass coverslips until they attained about 75% confluency. After appropriate treatments, cells were washed twice in 1X-PBS and fixed, permeabilized and immunostained for Nm23-H1, cdc42, Rac1, RhoA, and total FAK as described previously (32). The slides were viewed and imaged using a Leica SP8 confocal laser-scanning microscope.

**Wound-healing assays:** Cell migration was determined using the scratch wound healing assay. HCC1806 and BT549 cells were seeded on 24-well plates (Corning Inc.). Cells were transiently transfected as described above. When the cells reached approximately 90% confluence, a wound was generated by scratching the cell monolayer using a 200 µl pipette tip and then washed with PBS for removal of cell debris. Complete media was added to the cells and wound areas were photographed at 0h, 24h, and 48h post-scratch using an Olympus inverted microscope connected to a DXM1200 digital camera (Nikon, Tokyo, Japan).

**Spheroid migration assays:** For migration assays, \(1.0 \times 10^4\) cells/well suspended in complete medium (or media containing 2.5% reconstituted Matrigel basement membrane, for BT549 cells) were seeded onto 1.0% agar-coated 96-well plates and cultured for 48 hours in a humidified atmosphere containing 5% CO\(_2\) at 37°C. After spheroids formed, cells were then transiently transfected with scramble control siRNA or Nme1 siRNA for 48 hours. Intact tumor spheroids were carefully transferred to six-well plates and cultured in complete media for 24 hours. Spheroids and migrated cells were fixed with 10% buffered formalin, stained with 0.05% crystal violet, and observed using a normal light microscope (20X) and Olympus DP-30BW digital camera. Images are representative of three independent experiments, each performed in triplicate.
**Matrigel Invasion assay:** Invasion assays were performed using Transwell chambers with 8-µm pore polycarbonate membrane inserts (Corning Inc., Corning, NY, USA). 50,000 cells/well were plated onto the upper chambers of Matrigel-coated inserts and allowed to invade for 24 hours. Fetal bovine serum and fibronectin were used in the lower chambers as chemoattractants. Non-invading cells were removed and invaded cells in the membrane were fixed in methanol, washed, and stained with 0.1% crystal violet. Inserts were washed, briefly air-dried, and mounted onto glass slides and coverslipped. Invaded cells were photographed using an inverted Olympus CKX41 microscope (20X magnification) attached to an Infinity 1 camera. Five fields were counted for each sample. Data represents the average of 3 independent experiments ± standard deviation (S.D.)

**RT-PCR:** Total RNA was extracted using the RNeasy purification kit (Qiagen, Valencia, CA 91355) and treated with DNase (Invitrogen). cDNA was prepared from total RNA using random primers and the Superscript III first strand synthesis Kit (Invitrogen). Relative levels of mRNA were determined by real-time quantitative PCR using an Eppendorf cycler and the TaqMan Universal PCR master Mix (Applied Biosystems, Carlsbad, CA). Primers for Nme-1 (Hs02621161_s1), cdc42 (Hs00918944_g1), Rac1 (Hs01902432_s1), RhoA (HS00357608_m1), FAK (Hs01056457_m1), and RPLPO (Hs99999902_m1) were obtained from Applied Biosystems (TaqMan Gene Expression Assays). Samples were normalized against the RPLPO internal control using the 2–∆∆Ct method, compared as arbitrary units, and represented as mean ± SD. Samples were performed in triplicate and experiments were repeated at least three times with reproducible results.
Statistical Analysis: Quantitative data from in vitro experiments are presented as mean ± SD of experiments repeated at least three times in triplicate. Differences among group means were analyzed using one-way ANOVA or unpaired Student’s t-test. Differences were considered significant at p<0.05.

Results:

Following Nm23-H1 knockdown using siRNA silencing, protein levels of Nm23-H1 and downstream targets were examined using Western blotting (Figure 2A). Nm23-H1 was effectively silenced in both epithelial and mesenchymal TNBC cell lines. Next, we wanted to determine the effects of Nm23-H1 silencing on the levels of downstream GTPase targets: Rho, Rac, and Cdc42 (Figure 2A). We found that inhibiting Nm23-H1 expression resulted in increased protein levels of both Rho and Rac1 but decreased expression of Cdc42 protein in HCC1806 epithelial cells. Conversely, Nm23-H1 silencing in the mesenchymal cell line, BT549, resulted in decreased levels of Rho and Rac, whereas Cdc42 protein levels increased. Expression of the motility marker FAK was also examined in both epithelial and mesenchymal cells. Total FAK levels did not change between the control and the knockdown. Nm23-H1 inhibition resulted in increased activation of pFAK in HCC1806 cells and reduced expression of pFAK in BT549 control treatments (Figure 2A).

TNBC cells were examined for morphological changes following Nm23-H1 siRNA silencing (Figure 2B). Control HCC1806 cells appeared epithelial-like: rounded with cell-cell contact to neighboring cells. Following Nm23-H1 silencing, HCC1806 cells appeared to resemble a more mesenchymal phenotype (i.e., low cell-cell contact and
fibroblastic in appearance). BT549 control cells appeared mesenchymal-like; however, when Nm23-H1 was silenced in these cells, their morphology appeared more rounded and epithelial-like (Figure 2B).

Quantitative RT-PCR was performed in order to determine the effects of silencing Nm23-H1 mRNA on Cdc42, Rac1, RhoA, and FAK transcription (Figure 2C). In both cell lines, Nm23-H1 transcription was successfully reduced in Nme-1 siRNA transfected cells compared to the control cells. In the HCC1806 cell line, Nm23-H1 knockdown decreased Cdc42 mRNA levels (Figure 2C), which was consistent with the observed decrease in Cdc42 protein expression in these cells following siRNA treatments (Figure 2A). Conversely, Rac-1, RhoA, and FAK transcripts were decreased in HCC1806 cells, which is opposite of Western blot data demonstrating an increase in protein expression of these markers in response to Nm23-H1 inhibition (Figure 2A). For the mesenchymal cell line, BT549, we observed increased mRNA levels of Cdc42 (which was consistent with Western blot data, Figure 2B); however, Rac-1, RhoA, and FAK transcript levels were also increased in the knockdown cells compared to the control cells, which was opposite of the protein expression levels (Figure 2C). We are not quite sure how to explain the differences in transcript expression and protein expression levels of Rac1, RhoA, and FAK in response to Nm23-H1 siRNA-mediated inhibition.

Next, we wanted to examine changes in protein expression levels and cellular localization in response to siRNA treatments using immunocytochemistry analyses (Figure 3). In both HCC1806 and BT549, Nm23-H1 was localized in the nucleus and cytoplasm, and antibody staining demonstrated that Nm23-H1 had been effectively silenced. Cdc42, Rac1, RhoA, and FAK protein expressions were observed only in the
cytoplasm of HCC1806 and BT549 cells. Following silencing of Nm23-H1 in HCC1806 cells, we observed decreased cytoplasmic protein expression of Cdc42 and increased expression of Rac1, RhoA, and FAK compared to the control (Figure 3). In BT549, the opposite trend was observed. Following siRNA silencing, Cdc42 increased, whereas Rac1, RhoA, and FAK all decreased. No clear change in cellular localization was noted for either protein.

Wound-healing assays were then performed in order to establish the role of Nm23-H1 on TNBC cell migration (Figure 4A). Nm23-H1-inhibited HCC1806 cells migrated much quicker than the control, as evidenced by an increased closure rate of the wound. BT549 demonstrated the opposite trend; the wound closed much quicker in the control cells compared to the knockdown, which suggests that the control cells have increased migratory capacity. To further examine changes in migration rates as a result of Nm23-H1 knockdown, spheroid migration assays were also conducted (Figure 4A). The same trend found in the wound-healing assays was again observed. In HCC1806 cells, when Nm23-H1 was silenced, we noted an increase in migration away from the spheroid, which indicates increased migratory potential. In the BT549 cell line, decreased migration from the spheroid was seen in knockdown cells when compared to the control cells.

Finally, the effects of Nm23-H1 on the invasive potential of TNBC cells were examined using Matrigel invasion assays. As shown in Figure 4B, in HCC1806 cells when Nm23-H1 was knocked down, the number of cells that invaded the matrix increased significantly compared to the control. However, the opposite was seen in
BT549 cells (Figure 4C). Fewer Nm23-H1 siRNA cells invaded the matrix in this cell line as compared to the control.
CHAPTER 4: Effects of Nm23-H1 overexpression on TNBC cells

**Specific Aim 3:** Investigating the functional effects of increasing Nm23-H1 expression using megestrol acetate (MA)

**Rationale:** Since reducing Nm23-H1 protein levels results in tissue specific changes in two morphologically different TNBC cell lines, we hypothesized that increasing Nm23-H1 expression using the clinical drug, MA, would produce the opposite effects. By treating cells with MA, we were able to further correlate whether increased Nm23-H1 serves paradoxical roles in TNBC cell lines.

**Methods:**

**SRB Survival Assays:** HCC1806 and BT549 cells were seeded at a density of 5000 cells/well in 96-well plates and grown overnight before treatment with MA (Sigma-Aldrich) in complete culture media for 72 hours. Cell viability was assessed using sulforhodamine-B (SRB) assays following procedures previously described (33).

**Dose Curve:** HCC1806 and BT549 cells were seeded in 6-well plates and cultured in triplicate in DMEM media with or without MA for 24 hours. The concentrations of MA treatment were 0, 25, 50, 100, 200, or 400 µM. Total lysates were collected and analyzed via Western blots as described in Specific Aim 1.

**Time Course Treatments:** HCC1806 and BT549 cells were seeded in 6-well plates and cultured in triplicate in DMEM media with or without 130 µM MA for 0h, 1h, 2h, 6h, 10h, or 24h time points. Total lysates were collected and analyzed via Western blots as described in Specific Aim 1.
**Wound-healing assays:** Following treatment with MA, wound-healing assays were performed as described in Chapter 3 (Specific Aim 2).

**Spheroid Migration assays:** Following treatment with MA, spheroid migration assays were performed as described in Chapter 3 (Specific Aim 2).

**Matrigel Invasion Assays:** Following treatment with MA, Matrigel invasion assays were performed as described in Chapter 3 (Specific Aim 2).

**GTPase Activity Assays:** The RhoA/Rac1/Cdc42 Activation Assay Combo Biochem Kit (Cytoskeleton, Inc) was used according to manufacturer’s instructions to determine the active levels of GTPases in response to MA treatments. HCC1806 and BT549 cells cultured with or without MA were separately harvested and suspended with lysis buffer. Supernatant of each sample was collected by centrifugation (10,000 x g) at 4°C for 1 minute then immediately snap-frozen in liquid nitrogen. The protein sample concentrations were determined by bicinchonicic acid (BCA) assay and adjusted to a final amount of 300µg in a volume of 200µl. Then, 10µl of Pak-PBD-conjugated beads (for Rac1 and Cdc42 activation assays) or 30µl of Rhotekin-RBD-conjugated beads (for RhoA activation assays) was added and each sample was continuously rotated at 4°C for 1 hour. The incubated samples were further washed with lysis buffer and collected by centrifugation (5,000 x g) at 4°C for 1 minute. After 2X Laemmli sample buffer was added to each sample, they were boiled for 5 min and analyzed by SDS-PAGE and Western blotting to determine the Rac1-GTPase, Cdc42-GTPase, and RhoA-GTPase activity.
**Results:**

Following treatment with megestrol acetate (MA), cell viability was assessed using SRB survival assays (Figure 5A). In both HCC1806 and BT549 cell lines, cell survival did not significantly decrease despite increasing concentrations of the drug, which suggests that Nm23-H1 does not play a significant role in TNBC cell survival.

In order to determine an effective dose of MA to use for experimentation, a dose curve was performed (Figure 5B). Nm23-H1 protein levels were analyzed by Western blotting following increased dosing of MA. Figure 5B shows that in both cell lines, MA concentrations as low as 25 µM effectively increased Nm23-H1 protein expression, with optimal doses between 100-400 µM. Based on these results, 130 µM MA was used for both cell lines in subsequent experiments.

Next, an MA time course treatment was performed to determine the optimal time for treating TNBC cells for subsequent experiments. Following treatment with 130 µM of MA over time (0h, 1h, 2h, 6h, 10h, and 24h), protein expression levels of Nm23-H1, the Rho GTPases, and FAK were analyzed via immunoblotting (Figure 5C). MA treatments increased Nm23-H1 protein levels in a time-dependent manner in both cell lines. In HCC1806 cells, Cdc42 expression increased between 1h and 2h post-treatment with MA, but decreased back to basal levels 6h-24h after treatments. However, as expected, RhoA and Rac1 protein expression both decreased in a time-dependent manner in HCC1806 cells. pFAK protein expression initially decreased in HCC1806 cells following MA treatments; however, after 10 hours, expression began to increase. Total FAK expression in HCC1806 initially increased and by 6h began to decrease. In the mesenchymal BT549 cell line, we also observed a time-dependent increase in Nm23-H1 protein levels, which
was sustained 24h following treatment with MA (Figure 5C). In this cell line, MA treatment decreased Cdc42 expression over time. The opposite is seen for RhoA, Rac1, pFAK and total FAK, all of which increased following MA treatment (Figure 5C). Based on Nm23-H1 protein levels in this experiment, 4 hours was selected as the desired MA time treatment for subsequent experiments.

The migratory capacity of TNBC cell lines was also assessed following MA treatment. In HCC1806, MA-treated cells migrated much slower than the control cells, as shown by wound-healing and spheroid migration assays (Figure 6A). In BT549 cells, the opposite was observed. Both wound-healing assays and spheroid migration assays demonstrated MA-treated cells invaded the wound at an increased rate compared to the control cells (Figure 6A).

The effects of MA treatment on invasion were analyzed by Matrigel invasion assays (Figure 6B). When HCC1806 cells were treated with MA, we observed a statistically significant decrease in the number of cells that invaded the matrix. Conversely, following MA treatment in BT549 cells, a statistically significant increase in the number of cells invading the matrix was observed. These data confirm that Nm23-H1 plays an important but different role in TNBC cell migration and invasion and is dependent on the morphological subtype.

Finally, we analyzed the effects of MA treatment on the activity of Cdc42, RhoA, and Rac1 GTPases. (Figure 6C). The active (GTP-bound) and total protein levels were examined by activation pull-down assays and Western blotting following MA treatments. These studies showed that MA stimulation in HCC1806 cells did not have an effect on the levels of the GTP-bound form of Cdc42. However, there was a slight decrease in
active RhoA and Rac1 levels in response to MA. Total Cdc42, RhoA, and Rac1 remained unchanged. In BT549 cells, protein levels of Cdc42-GTP decreased following MA treatment. The GTP-bound form of Rho was increased in BT549 cells; however, active Rac1 was surprisingly reduced. Total protein levels of Cdc42, RhoA, and Rac1 did not change following MA treatment.

CHAPTER 5: Discussion and Conclusions

Specific Aim 1:

The Pathway Studio Program demonstrated that previous studies have established a link between Nm23-H1 and two of the small Rho GTPases of interest: Rac1 and Cdc42. Nm23-H1 has been shown to increase levels of Cdc42 and decrease levels of Rac1. However, a link between Nm23-H1 expression and Cdc42, Rac1, and particularly RhoA activities has not been established in TNBC tissues. One novel aspect of this study was the examination of Nm23-H1’s effect on RhoA in TNBC cells.

In order to characterize the role of Nm23-H1 expression in TNBC cells, protein levels of Nm23-H1, the Rho GTPases, and FAK were examined via Western blotting in an epithelial and a mesenchymal human TNBC cell line. All of the proteins examined were present in these cell lines in varying levels, except for active FAK (pFAK), which was absent in the HCC1806 cell line. Phosphorylated FAK functions as a marker of migration by mediating cell shape, adhesion, and motility, which may explain why higher levels are seen in the more migratory mesenchymal phenotypes (34). The epithelial cell line may express higher protein levels of Cdc42 because this protein plays a crucial role in sustaining cell polarity by maintaining adherens junctions (25), unlike in mesenchymal
cells. Once epithelial cells transition to mesenchymal phenotypes during EMT, they lose their polarity, perhaps due, in part, to decreased expression of Cdc42 protein. This loss in cell polarity is typically accompanied by an increase migratory potential, which may be effected by increased FAK activation. Our data clearly demonstrates that epithelial TNBC cells possess higher levels of Cdc42 and lower levels of active FAK compared to mesenchymal TNBC cells.

Specific Aim 2:

Morphologically distinct HCC1806 (non-metastatic) and BT549 (metastatic) cell lines were used to examine the functional significance of the tumor suppressor protein Nm23-H1 in TNBC subtypes. Surprisingly, our data suggests that Nm23-H1 may have distinct, opposite roles in different TNBC subtypes. Specifically, Nm23-H1 functions as a tumor suppressor in the less aggressive, epithelial HCC1806 cell line. When Nm23-H1 protein levels were reduced in HCC1806 cells, the cell line transitioned to a more mesenchymal phenotype (i.e., undergoes EMT) as evidenced by changes in morphology. These morphological changes were accompanied by reduced expression of Cdc42 and increased levels of RhoA, Rac1, and active FAK. The decrease in Cdc42 following Nm23-H1 knockdown supports the observation that the HCC1806 cells lose epithelial cell polarity, thereby becoming more mesenchymal-like. Furthermore, Rho and Rac1 proteins have been shown to enhance cell migration (25) and increased pFAK is associated with the disintegration of adhesion sites, which renders cell more motile (34). Interestingly, the opposite trends were observed when Nm23-H1 was silenced in the mesenchymal cell line, BT549. Our data suggests that Nm23-H1 may function as an oncogene in metastatic TNBC cells and silencing the gene exerts metastasis suppressive
effects. Following Nm23-H1 silencing in BT549 cells, Cdc42 protein levels increased and RhoA, Rac-1, and pFAK levels decreased. These changes in protein expression levels were accompanied by morphological changes, which suggest a mesenchymal-epithelial transition (MET). These results are in line with a few reports that demonstrate that increased Nm23-H1 levels correlate with aggressive tumor features in other aggressive tumor types (24).

The effects of Nm23-H1 expression on Rho GTPase and FAK transcription activity in TNBC cells were inconclusive. Although Nm23-H1-specific siRNA inhibition effectively reduced expression of the protein in both cell lines, the resulting data contradicted the changes that were noted in Western blotting experiments. Cdc42 transcripts and proteins were increased in the HCC1806 cells and decreased in BT549 cells. However, changes in Rac1, RhoA, and FAK mRNA levels were opposite of what was expected based on results of Western blotting and morphological examination. This could be due to the fact that protein levels may be a better predictor of outcome if not all mRNA is translated into protein. In order to better understand these results, RT-PCR would need to be repeated, however time constraints did not allow this to be done.

Data from immunocytochemical assays further validate that Nm23-H1 has tumor suppressive activity in HCC1806 and oncogenic activity in BT549. Similar to Western blot analyses, Nm23-H1 inhibition resulted in paradoxical changes in Rho GTPases and FAK expression in HCC1806 and BT549 TNBC cell lines. Following Nm23-H1 siRNA inhibition, Cdc42 protein expression levels decreased in HCC1806 cells but increased in BT549 cells. Additionally, expression of RhoA and Rac1 GTPases and FAK proteins increased in the HCC1806 knockdown cells and decreased in the BT549 knockdown. No
clear changes in the cellular locations of Rho GTPases or FAK were observed in either cell line, which suggests that Nm23-H1 does not alters the subcellular locations of these markers.

Wound-healing, spheroid, and invasion assays also demonstrated the tissue-specific roles of Nm23-H1 in the migratory and invasive abilities of TNBC cells. Nm23-H1 silencing resulted in a significant increase in the migratory potential of HCC1806 cells but a significant decrease in migration of BT549 cells. In addition, Matrigel invasion assays showed increased invasive potential in HCC1806 and decreased invasive potential in BT549 following Nm23-H1 knockdown. Again, these data suggest that Nm23-H1 plays important and distinctive functional roles in epithelial and mesenchymal TNBC phenotypes.

**Specific Aim 3:**

Both cell lines were treated with megestrol acetate (MA) in order to assess the effects of increasing Nm23-H1 gene expression on the two subtypes of TNBC. MA, a progestin that indirectly reduces both estrogen and androgen levels, is one of the oldest compounds that has been used to treat metastatic breast cancer, and the drug is still used today (35). MA is an analogue of medroxyprogesterone acetate (MPA), which has been shown to increase Nm23 levels in triple-negative breast cancer (36). Due to MA’s similarity to MPA, MA was chosen as a drug treatment for both HCC1806 and BT549 TNBC cells.

SRB survival assays demonstrated that MA has no effect on survival in either epithelial or mesenchymal TNBC cell lines. MA increases Nm23-H1 gene expression in a dose-dependent and time-dependent manner in both cell lines.
Once again, Nm23-H1 appears to be acting as a tumor suppressor in HCC1806 but an oncogene in BT549. In the epithelial cell line, Rho, Rac1, pFAK, and total FAK decreased following MA treatment, while Cdc42 seems to transiently increase and then decrease back to basal levels. On the other hand, in the mesenchymal cell line, increasing Nm23-H1 protein levels resulted in an increase in Rho, Rac1, pFAK, and total FAK expression.

The effects of MA treatment on migration and invasion of the different TNBC cell types were also examined by wound-healing, spheroid migration, and Matrigel invasion assays. Our data revealed that treating HCC1806 with MA renders them less migratory, as evidenced by a slower closure of the wound and less migration from the spheroid. In the epithelial HCC1806 cell line, Nm23-H1 appears to be exhibiting its metastasis suppressive effects, which is in strong agreement with data obtained in Nm23-H1 inhibition studies. Likewise, in MA-treated BT549, Nm23-H1 exerted oncogenic effects, including increased cell migration and invasion compared to the control, which is consistent with data obtained in Nm23-H1 inhibition studies.

The effects of MA treatment on the activity of the Rho GTPases remains a bit inconclusive. Based on previous data, we expected to see an increase in active Cdc42 and decreased levels of active RhoA and Rac1 in HCC1806 cells and decreased GTP-bound Cdc42 and increased active forms of RhoA and Rac1 in BT549 cells. However, not much change was seen in protein levels of the active, GTP bound form of Cdc42 in HCC1806, but decreased GTP-bound Cdc42 in BT549 suggests that these cells are further losing polarity. The decreased expression of GTP-bound form of Rho following MA treatment in HCC1806 further demonstrates the suppressive function of Nm23-H1 in epithelial
cells. Conversely, the increase of Rho in BT549 supports the oncogenic function of Nm23-H1 in mesenchymal cells. These experiments were only performed twice and time and money did not allow us to further evaluate the effects of MA treatments on the active forms of these proteins.

**Conclusions**

Nm23-H1 was identified as the first metastasis suppressor that can prevent the formation of metastases while having no effect on the size of the primary tumor. Nm23-H1 has been shown to suppress metastases in several types of cancer, however, the mechanism by which it functions and its role in TNBC still remains unknown (24). Although Nm23-H1 has been shown to suppress metastases, this may not be the case for all types of cancer. In thyroid cancers, neuroblastoma, and osteosarcoma, increased gene expression of Nm23-H1 has been associated with poorer prognoses (24). Our data strongly suggests that in epithelial TNBC cells, Nm23-H1 functions as a metastasis suppressor; however, Nm23-H1 serves as an oncogene in mesenchymal TNBC cells. We therefore propose that in TNBC, Nm23-H1 plays a tissue specific role.

Initially, we thought that Nm23-H1 would be a very interesting potential target to investigate in TNBC due to the possibility of its metastasis suppressive effects and the highly metastatic behavior of a majority of TNBCs. If Nm23-H1 served as a metastasis suppressor in TNBC as we originally hypothesized, targeting this gene could prevent the most deadly aspect of all cancers, metastasis. However, our data suggests that in TNBC, Nm23-H1 does not always exert suppressive effects, but instead, may paradoxically exert oncogenic effects depending on tissue phenotype. Both silencing Nm23-H1 and increasing its expression by MA treatment demonstrated that in epithelial cells, Nm23-H1
acts as a metastasis suppressor, but in cells of the mesenchymal phenotype, it acts as an oncogene.

Clinically, understanding Nm23-H1’s paradoxical role in TNBC could be of great importance. TNBC’s are an extremely heterogeneous class of tumors, and our data suggests that targeting Nm23-H1 may serve as a potential therapeutic target in some TNBC tumors, but may actually worsen prognosis in others. Characterizing tumor phenotypes prior to Nm23-H1 targeted therapy may be essential to assessing whether this could serve as a viable option. However, in epithelial TNBC cells, this study suggests that targeting Nm23-H1 could prevent metastasis by decreasing Rho, Rac1, and pFAK, while increasing epithelial cell polarity via increasing Cdc42 expression. Targeting Nm23-H1 in a subset of epithelial TNBCs could serve as a novel therapeutic strategy and prevent mortality associated with TNBC metastasis. This report is the first to define the specific mechanisms and paradoxical effects of Nm23-H1 in TNBC cells and to provide a strong rationale for further investigating its potential as a viable therapeutic option for these aggressive and deadly tumor types.
Figure 1: Expression profile of Nm23-H1 and downstream targets in TNBCs. (A) Analysis of interactions of Nm23-H1 that exist in published scientific literature with RhoA, Rac1, and Cdc42 performed by Pathway Studio. (B) Endogenous expression of Nm23-H1, Rho, Rac1, Cdc42, pFAK, and FAK analyzed via Western blot analysis in a panel of human TNBC cells. β-actin was used as a loading control.
**Figure 2: Nm23-H1 silencing in TNBC cell lines.** (A) Western blot analysis of Nm23-H1, Rho GTPases (RhoA, Rac1, Cdc42), and motility markers (FAK and pFAK) following siRNA silencing using specific antibodies. β-actin was used as a loading control. (B) Morphological changes of TNBC cell lines examined in both siRNA control and siRNA silenced cells. (C) Related mRNA expression levels of Nm23-H1, Cdc42, Rac1, RhoA, and FAK in HCC1806 and BT549 control and Nm23-H1 siRNA silenced cell lines were detected by TaqMan quantitative RT-PCR and normalized to RPLPO.
Figure 3: ICC analysis of Nm23-H1 siRNA. Immunofluorescent analysis was performed on HCC1806 and BT549 cells transiently transfected with control siRNA or Nm23-H1 siRNA. Cells were labeled with Nm23-H1, Cdc42, Rac1, RhoA, and FAK antibodies (shown in green). DAPI (blue) was used to identify the nuclei.
Figure 4: Effects of Nm23-H1 silencing on TNBC migration and invasion. (A) The migratory ability of HCC1806 and BT549 cell lines was investigated by scratch assays for cells transiently transfected with control and Nm23-H1 siRNA. Spheroid cell migration assays were also performed to examine the migratory potential of both control and siRNA silenced HCC1806 and BT549 cell lines. Matrigel invasion assays were used to measure the invasive potential of (B) HCC1806 and (C) BT549 cells transiently transfected with control and Nm23-H1 siRNA.
Figure 5: Effects of MA on TNBC cell survival and Nm23-H1 signaling. (A) HCC1806 and BT549 cells were treated with 2-fold serial dilutions of MA and analyzed by SRB assays. (B) Nm23-H1 expression was examined by Western blotting in HCC1806 and BT549 cells following treatments with indicated doses of MA for 24h. (C) HCC1806 and BT549 cell lines were treated with 130 µM MA for various time points (0h, 1h, 2h, 6h, 10h, and 24h). Total cell lysates were analyzed via Western blotting for protein levels of Nm23-H1, Cdc42, RhoA, Rac1, pFAK, and FAK. β-actin was used as a loading control.
Figure 6: Effects of Nm23-H1 overexpression on TNBC migration, invasion, and GTPase activity. (A) Migration potential of HCC1806 and BT549 cell lines were investigated by scratch assays for both control and cells treated with 130 µM of MA for indicated time periods. Spheroid cell migration assays were performed to examine the migratory potential of both control and MA treated HCC1806 and BT549 cell lines. (B) Matrigel invasion assays were performed on control and MA treated TNBC cells to measure invasive potential. (C) Rho GTPase activity assays were performed to measure both GTP-bound and total protein levels and analyzed via Western Blotting for TNBC cell lines.
References: