MENA Confers Resistance to Paclitaxel in Triple-Negative Breast Cancer

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Abstract

Taxane therapy remains the standard of care for triple-negative breast cancer. However, high frequencies of recurrence and progression in treated patients indicate that metastatic breast cancer cells can acquire resistance to this drug. The actin regulatory protein MENA and particularly its invasive isoform, MENAINV, are established drivers of metastasis. MENAINV expression is significantly correlated with metastasis and poor outcome in human patients with breast cancer. We investigated whether MENA isoforms might play a role in driving resistance to chemotherapy. We find that both MENA and MENAINV confer resistance to the taxane paclitaxel, but not to the widely used DNA-damaging agents doxorubicin or cisplatin. Furthermore, paclitaxel treatment does not attenuate growth of MENAINV-driven metastatic lesions. Mechanistically, MENA isoform expression alters the ratio of dynamic and stable microtubule populations in paclitaxel-treated cells. MENA expression also increases MAPK signaling in response to paclitaxel treatment. Decreasing ERK phosphorylation by co-treatment with MEK inhibitor restored paclitaxel sensitivity by driving microtubule stabilization in MENA isoform–expressing cells. Our results reveal a novel mechanism of taxane resistance in highly metastatic breast cancer cells and identify a combination therapy to overcome such resistance. Mol Cancer Ther; 16(1); 143–55. ©2016 AACR.

Introduction

While the number of targeted agents in clinical trials for metastatic breast cancer continues to increase, chemotherapy remains the standard of care for this disease, particularly for triple-negative breast cancer (TNBC). TNBC, one of the most aggressive breast cancer subtypes (1), is defined by the lack of expression of both estrogen and progestrone receptors as well as low levels of HER2, accounts for 15% of breast tumors. TNBC is associated with a poorer prognosis along with a greater risk of recurrence and metastasis (2, 3). Platinum agents, taxanes and anthracyclines, are used in mono- or poly-chemotherapy as front-line treatment, especially in the context of metastatic disease (4). Antimitotic chemotherapy agents generally target proliferating cancer cells, with platinum agents such as cisplatin and anthracyclines, including doxorubicin primarily functioning through direct interaction with DNA and subsequent interference with its replication. In contrast, taxanes such as paclitaxel lead to mitotic catastrophe by stabilizing microtubules and inhibiting their disassembly during metaphase, thus leading to mitotic arrest and cell death (5, 6). The benefits of cytotoxic chemotherapy for TNBC are clear; nevertheless, response rates are low, and more than 50% of patients with TNBC become resistant to chemotherapy typically by 6 to 10 months (7).

A number of cellular processes are known to drive chemoresistance, which can arise from both cell-intrinsic mechanisms and tumor microenvironment–driven external survival signals (8). First, changes in expression of the ATP-binding cassette (ABC) superfamily of transporters, in particular P-gp/MDR1, have been shown to be involved in paclitaxel resistance (9). While in vitro and preclinical studies showed increased cell death with dual chemotherapy and MDR1 inhibitor, the first and second generation of inhibitors both failed in clinical trials due to safety and efficacy issues (9). Second, mutations in a drug’s cellular targets, β-tubulin in the case of taxanes, can drive cell-intrinsic resistance by impairing paclitaxel binding, increasing microtubule dynamics, and blocking taxane-induced G2–M arrest (10). Third, changes in expression or function of proteins of the apoptosis pathways, such as caspase-3 or Bcl-2, have also been implicated in taxane resistance (11, 12). Chemotherapy-induced proapoptotic signals can be counteracted by constitutive activation of the prosurvival PI3K/Akt/mTOR or RAF/RAS/MEK MAPK signaling pathways (13). Paclitaxel treatment can induce MAPK activation to attenuate microtubule stabilization (10), and multiple studies have demonstrated that co-treatment with paclitaxel and a MEK inhibitor can increase cancer cell death (14). Much of our understanding of these and other mechanisms of taxane resistance is derived from studies focused on cell survival and proliferation. The question of how such mechanisms of resistance are associated with cell invasion and metastatic disease itself remains understudied.
One key driver of breast cancer metastasis is MENA (also known as ENAH or hMena), a member of the Ena/VASP family of actin elongation factors that is upregulated in various cancer types, including breast cancer (15). MENA deficiency in the PyMT mouse model of breast cancer slows tumor progression and decreases metastasis (16), and stable depletion of MENA in the human breast adenocarcinoma MDA-MB-231 cells significantly decreases metastatic burden from orthotopic xenograft tumors (17). In addition to a broadly expressed 80-kDa MENA isoform, multiple other MENA protein isoforms can arise in some cell types from changes in the inclusion of 5 alternatively spliced exons in the MENA mRNA (18–20). In human patients with breast cancer and in mouse breast cancer models, changes in expression of 2 functionally distinct isoforms, MENA11a and MENAINV, are linked to metastatic tumor cell phenotypes and to patient outcomes. MENA11a expression is high in normal epithelial tissues and in epithelial-like cells in breast cancer cells in vitro and in vivo (19, 21) but is low in invasive tumor cell subpopulations (22). Men11a suppresses tumor cell migration and invasion and promotes cohesive tumor morphology (19, 21, 23). While expression levels of either MENA or MENA11a alone do not correlate breast cancer patient outcome (17, 24), patients with high Menainv, a measure of the total MenA levels minus MenA11a levels, have poor disease-specific survival (24, 25).

In contrast to MENA11a, MENAINV expression is significantly higher in metastatic than in nonmetastatic human breast tumors, and high levels of MENAINV, but not MENA, are associated with increased metastasis and poor outcome in multiple human breast cancer cohorts (17). MENAINV is highly upregulated in invasive tumor cell subpopulations in vitro but found only at trace levels in even highly aggressive breast cancer cell lines in culture (22, 26). Expression of MENAINV in breast tumor cells renders them more sensitive to multiple proinvasive growth factors (27, 28) and increases their ability to migrate toward higher concentration of fibronectin, which is especially abundant near blood vessels (17). As a consequence, cells expressing MENAINV are more invasive and are highly metastatic. MENAINV has been shown to drive resistance to targeted kinase inhibitors in growth factor–elicited motility responses (28); however, its role in regulating responses to chemotherapeutic drugs has never been studied. Chemotherapy regimens containing taxanes are the standard of care for patients with metastatic breast cancer (29). Given the fact that both MENAINV and MENA are expressed in metastatic breast tumors and drive prometastatic phenotypes, we wondered whether expression of MENA and MENAINV affected tumor cell responses to chemotherapy, and how, in turn, standard-of-care cytotoxic therapy might influence MENA/MENAINV-driven metastasis. We show, using in vitro and in vivo models, that highly metastatic cells expressing MENA or MENAINV confer paclitaxel resistance, by preventing the microtubule stabilization via activation of the MAPK signaling cascade. Our findings reveal a novel mechanism by which highly metastatic breast cancer cells can become resistant to taxanes.

Materials and Methods

Antibodies and drugs

**Antibodies.** The anti-MENA and anti-MENAINV antibodies were generated in the laboratory and previously described (26, 30): anti-tubulin (Sigma, DM1A), anti-tubulin detyrosinated or Glutubulin (Millipore, AB3201), anti-tubulin tyrosinated (Millipore, ABT171), anti-pERK Y204 (Santa Cruz, sc7383), anti-GAPDH (Sigma, G9545), anti-Ki67 (BD Biosciences), cleaved Caspase-3 (BD Biosciences), and anti-pAkt473 ( CST).

**Drugs.** Docetaxel, cisplatin and paclitaxel (Sigma), doxorubicin, and gefitinib were the drugs used. For *in vitro* experiment, drugs were diluted in cell culture media with 1% of DMSO. Vehicle control correspond to the drugs used. For *in vivo* experiment, drugs were diluted in cell culture media with 1% of DMSO (no drug). PD0325901 MEK inhibitor (LC Labs), MDR1 inhibitor HM30181 (100 nmol/L) gift from the Weissleder Laboratory (MGH; ref. 31).

**Cell culture**

MDA-MB-231 cells were purchased directly from the ATCC in June 2012, where cell lines are authenticated by STR profiling. These cells were not reauthenticated by our laboratory and were cultured in DMEM with 10% FBS (Hyclone). Cell line generation and FACS were performed as previously described (32). Cell lines show an 8- to 10-fold overexpression relative to endogenous MENA and are labeled 231-Control, 231-MENA, and 231-MENAINV (17). SUM159 cells were obtained from Joan Brugge laboratory at Harvard Medical School (January 2011) and were not reauthenticated in our laboratory. SUM159 cells were cultured according to the ATCC protocols. T47D cells were purchased at ATCC, where cell lines are authenticated by STR profiling. They were cultured according to the manufacturer's protocol and not reauthenticated in our laboratory. Stable knockdown cell lines (T47D) were generated using a retroviral vector to express an mir30-based shRNA sequence 'CAGAAGACATCGCCCGTTAA' targeting a sequence shared among all known Mena mRNA isoforms. Western blot analysis, detected using an anti-Mena monoclonal that recognizes an epitope shared in all known Mena mRNA isoforms. Western blot analysis, detected using an anti-Mena monoclonal that recognizes an epitope shared in all known Mena mRNA isoforms. Western blot analysis, detected using an anti-Mena monoclonal that recognizes an epitope shared in all known Mena mRNA isoforms.

**Cell viability assay**

Cell viability assays were performed in a 96-well plate. Five thousand cells were plated per well and treated with drug 24 hours later. Cell viability was assessed 72 hours later using the PrestoBlue Cell Viability Reagent (Life Technologies), according to manufacturer's protocol. Fluorescence was measured and normalized to cells exposed to vehicle. The activity area was calculated from dose–response plots using MATLAB. All measurements were repeated in triplicates.

**Xenograft tumor generation and in vivo chemotherapy treatment**

Animal experiments were approved by the MIT Division of Comparative Medicine. Two million MDA-MB-231 cells expressing different MENA isoforms (in PBS and 20% collagen I) were injected into the fourth right mammary fat pad of 6-week-old female NOD/SCID mice (Taconic). When the tumors reached 1 cm in diameter, mice were treated every 5 days with 3 doses of paclitaxel at 10 mg/kg in 1% DMSO, 3% PEG (MW 400), 1% Tween-80 in PBS by intraperitoneal injection. In parallel, mice...
were treated with only 1% DMSO, 3% PEG (MW 400), 1% Tween-80 in PBS as a vehicle control. One day after the last injection, tumors were measured and mice were used for intravital imaging and then sacrificed. Their tumors and lungs were fixed in 10% formalin overnight and their bone marrow was collected using PBS and cultured in DMEM with 10% FBS. The number of tumor cell colonies in cultured bone marrow was counted 1 month after collection. The number of metastasis in each lobe of the lung was counted from lung hematoxylin and eosin (H&E)-stained sections visualized by light microscopy and counted by 2 blinded individuals. Each tumor group contained 3 to 5 mice.

Intravital imaging

Intravital multiphoton imaging was performed as described previously (17) using a 25× 1.05 NA water immersion objective with correction lens. After exposing the tumor with a skin flap surgery, 30-minute movies were captured. The number of motile cells in each field of view was counted in ten 30-minute time-lapse movies using Imagel. Motile cells were cells that showed any displacement of the nucleus and cell protrusion activity. Data were pooled from 2 to 4 mice per tumor group, with 4 to 10 fields imaged per mouse.

Western blot

Cells were lysed in 25 mmol/L Tris, 150 mmol/L NaCl, 10% glycerol, 1% NP-40, and 0.5 mol/L EDTA with a Mini-Complete protease inhibitor (Roche) and a phosphatase inhibitor cocktail (PhosSTOP, Roche) at 4°C for 20 minutes. Protein lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blocked with Odyssey Blocking Buffer (LiCor). Membranes were incubated with primary antibodies overnight at 4°C and LiCor secondary antibodies at room temperature for 1 hour. Protein level intensity was measured with Imagel.

Immunohistochemistry

Tumors dissected from NOD/SCID mice were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections (5 μm thick) were deparaffinized followed by antigen retrieval using Citra Plus solution (Biogenex). After treatment with 3% H2O2, sections were blocked with serum, incubated with primary antibodies overnight at 4°C and fluorescently labeled secondary antibodies at room temperature for 2 hours. Sections were stained using anti-MENA (1:500), biotinylated anti-MENAINV (1:500), anti-CC3 (1:200), anti-Ki67 (1:200), and DAPI. Fluorochochromes on secondary antibodies included AlexaFluor 488, 594, or 647 (Jackson ImmunoResearch). Sections were mounted in Fluoromount mounting media and imaged at room temperature. Z-series of images were taken on a DeltaVision microscope using Softworx acquisition, an Olympus 40× 1.3 NA plan apo objective and a Photometrics CoolSNAP HQ camera. At least 10 fields were captured for each tumor, with at least 3 tumors per tumor group.

Human breast cancer expression analysis

Data retrieval from The Cancer Genome Atlas (TCGA; ref. 34; Supplementary Fig. S1A and S1B) was explained by Oudin and colleagues (17). The data for MENAINV protein levels as measured by immunohistochemistry (IHC) are also described by Oudin and colleagues (17), from patient samples obtained from the study done by Wang and colleagues (35).

In vitro imaging

Glass-bottom dishes were coated with collagen at 0.1 mg/mL diluted in PBS for 1 hour at 37°C. Cells expressing different MENA isoform were treated with paclitaxel at 1, 10, or 100 nmol/L or vehicle and immediately plated on the glass-bottom dishes. Thirty-minutes later, cells were imaged overnight, with one image acquired every 10 minutes for 16 hours on a Nikon spinning disk with a 20× objective and an Andor/NeoZyla camera. Individual cells were manually tracked using Imagel and Manual Tracking plug-in. Data were analyzed using the chemotaxis tool developed by IBIDI. For analysis of time spent in cell division, we measured the time between when the mother cell first rounds up, to when both daughter cells have spread out on substrate. The percentage of successful cell divisions was quantified by counting the number of cell divisions that lead to 2 surviving daughter cells. Data are pooled from at least 50 cells tracked in 3 independent experiments.

Cell-cycle analysis

231-Control, 231-MENA, and 231-MENAINV cells were treated with paclitaxel at 10 or 100 nmol/L or vehicle. After 16 hours of treatment, cells were trypsinized, washed in cold PBS, centrifuged at 1,000 rpm for 3 minutes, resuspended in 1 mL of ice-cold PBS, fixed by adding 4 mL of ethanol at −20°C, and incubated at 4°C for 1 hour. After fixation, cells were washed with ice-cold PBS and centrifuged at 22,000 rpm for 20 minutes. DNA was stained using propidium iodide at 50 μg/mL and RNase A at 1 mg/mL for 30 minutes at 37°C. DNA content was measured on a FACSCalibur cytometer (Becton-Dickinson). Data were analyzed using Modfit software (Verity Software House), with appropriate gating on the FL2-A and FL2-W channels to exclude cell aggregates. Twenty-five thousand events were analyzed per sample.

Immunofluorescence

Glass-bottom dishes (Mattek) were coated with collagen at 0.1 mg/mL diluted in PBS and 50 μg/mL fibronectin for 1 hour at 37°C. Cells were plated for 1 hour and then treated with paclitaxel alone or in combination with MEKi for 24 hours. Cells were fixed for 20 minutes in 4% paraformaldehyde in PHEM buffer with 0.1% glutaraldehyde and then quenched with sodium borohydrate for 5 minutes. Cells were blocked with 2% BSA in TBS/0.1% Triton X-100 for 30 minutes and incubated with primary antibodies and then secondary antibodies for 1 hour each, at room temperature. Z-series of images were taken on an Applied Precision DeltaVision microscope using Softworx acquisition, an Olympus 60× 1.4 NA plan apo objective and a Photometrics CoolSNAP HQ camera. Images were deconvolved using Deltavision Softworx software and objective specific point spread function. Images were analyzed with Imagel, where the whole-cell intensity levels of Tyr-MT or Glu-MT were measured. Images are pooled from at least 3 independent experiments.

Microtubule length image analysis

Microtubule images were processed with (i) a filament reconstruction algorithm that selects bona fide filaments and (ii) a post-analysis that quantifies the properties of the microtubule network organization. For the filament reconstruction, briefly, the microtubule images were first filtered by multiple-scale steerable filter to enhance the curvilinear features. From the filtered images, the centerlines of possible filament fragments were detected and separated into high- and low-confidence sets. Some of
low-confidence filament fragments were linked to high-confidence fragments using iterative graph matching. The output of the reconstruction is a network of filaments each presented by an ordered chain of pixels and the local filament orientation. The microtubule length was calculated as the number of pixels converted to microns, per each identified filament. Overall, at least 2,000 microtubules were analyzed per condition, from at least 2 experiments.

**Results**

**MENA and MENAINV are associated with increased survival during paclitaxel treatment in vitro**

To investigate a potential role for MENA in resistance to chemotherapy in breast cancer, we first asked whether endogenous MENA and MENAINV expression levels were associated with paclitaxel resistance. MENA and MENAINV are widely expressed in all main breast cancer subtypes, as measured by mRNA from TCGA samples, as well as at the protein level by IHC (ref. 17; Supplementary Fig. S1A–S1C), with slightly higher expression in patients with Her2+ breast cancer. We then measured paclitaxel efficacy and quantified levels of endogenous MENA protein expression across cell lines from several human breast cancer types, including Luminal A (MDA-MB-175VI and T47D), HER2-positive (MDA-MB-453), and TNBC (SUM159, BT-20, MDA-MB-436, LM2, BT-549, MDA-MB-231; Fig. 1A and B, Supplementary Fig. S1D). In addition to the canonical 80-kDa MENA isoform, some of the cell lines used express other MENA isoforms endogenously, such as MENA11a, which is known to be expressed in epithelial-like cell lines including T47D cells and absent from mesenchymal-like cell lines including BT-549 and MDA-MB-231 cells. Under the conditions we used, MENA11a co-migrates with the 80-kDa MENA, detected with an antibody known to recognize all MENA isoforms, represents the total amount of 80-kDa MENA plus MENA11a in the cell lines that express both isoforms. There was a significant inverse correlation between paclitaxel efficacy, as measured by cell survival, and levels of endogenous MENA expression (Fig. 1C). To confirm that endogenously expressed MENA promotes paclitaxel resistance, we knocked down MENA in T47D cells, which normally express MENA and MENA11a (Supplementary Fig. S1E; refs. 19, 21). It is important to note that the shRNA used for these experiments targets a sequence common to all known MENA isoforms, thereby depleting...
MENA11a as well as MENA. Reducing all MENA isoform levels (>75%) in T47D cells renders them more sensitive to paclitaxel (Fig. 1D).

To study the role of MENA and MENAINV independently, we used a triple-negative breast adenocarcinoma cell line (MDA-MB-231), which endogenously expresses low levels of MENA and like other cultured breast cancer cell lines only trace levels of MENAINV_in vitro. As endogenous MENAINV expression is highly upregulated by aggressive tumor cells within the in vivo tumor microenvironment (17, 22, 26), we stably overexpressed GFP (231-Control), GFP-tagged MENA (231-MENA), or MENAINV (231-MENAINV) at equivalent levels in this cell line to match the

Figure 2.
MENA or MENAINV expression weakened paclitaxel effect on tumor growth in vivo. A, Tumors were generated by injection of 231-Control, 231-MENA, or 231-MENAINV cells in the mammary fat pad of NOD/SCID mice. When tumors reached 1 cm in diameter, mice were treated with paclitaxel every 5 days for 3 doses at 10 mg/kg intraperitoneally. Tumor volume was measured before and after treatment. B, Relative change in tumor volume after treatment with paclitaxel of tumors expressing the different GFP-tagged MENA isoforms. Data are presented as mean ± SEM for at least 9 mice in each group. Statistics determined by unpaired t test. **, P < 0.001; *, P < 0.01; *, P < 0.05. C, Representative images tumor sections from 231-Control, 231-MENA, and 231-MENAINV, treated with vehicle or paclitaxel, and stained for the proliferation marker Ki67 (green). Scale bar is 100 μm. D, Quantification of the Ki67 staining intensity in 231-Control, 231-MENA, and 231-MENAINV tumors, with and without paclitaxel treatment. E, Representative images tumor sections from 231-Control, 231-MENA, and 231-MENAINV, treated with vehicle or paclitaxel, and stained for the apoptosis marker cleaved caspase-3 (CC3; green). Scale bar is 100 μm. F, Quantification of the CC3 staining intensity in 231-Control, 231-MENA, and 231-MENAINV tumors, with and without paclitaxel treatment. Data are presented as mean ± SEM for at least 3 mice in each group, with at least 5 fields of view per tumor. Statistics determined by unpaired t test. ***, P < 0.001; **, P < 0.01; *, P < 0.05.
robust expression observed in vivo. We observed that the fraction of viable 231-MENA or 231-MENAINV cells was at least 65% higher than the fraction of viable 231-Control cells, after 72 hours of treatment with varying doses of paclitaxel (Fig. 1E). To investigate the specificity of the response, we also tested 2 other commonly used chemotherapeutics, doxorubicin and cisplatin, and found that neither MENA nor MENAINV expression affected the response to the different concentrations of either drug (Supplementary Fig. S1F and S1G). These experiments revealed that cell viability in the presence of high paclitaxel concentrations is decreased with low MENA expression and increased by ectopic expression of MENA or MENAINV. These data suggest that the increased levels of MENA isoforms observed in tumor cells during metastatic progression may contribute to paclitaxel resistance.

MENA isoform expression is associated with increased tumor growth in vivo during paclitaxel treatment

We then investigated whether MENA-associated paclitaxel resistance could also be observed in vivo. Xenograft tumors were generated by injecting MDA-MB-231 cells expressing MENA isoforms into the mammary fat pads of NOD/SCID mice. Mice were treated with paclitaxel once tumors reached 1 cm in diameter (Fig. 2A). Treatment with paclitaxel significantly decreased the growth of 231-Control tumors compared to mice treated with vehicle (Fig. 2B). However, the growth of 231-MENA or 231-MENAINV tumors was unaffected by paclitaxel treatment (Fig. 2B), thereby suggesting that MENA and MENAINV promote drug resistance in vivo.

The increased size of paclitaxel-treated 231-MENA and 231-MENAINV tumors could arise from elevated levels of proliferation, decreased levels of cell death, or both. We evaluated proliferation and apoptosis by quantifying the intensity of cells positive for Ki67 and CC3, respectively, by immunostaining. Although paclitaxel treatment decreased the amount of Ki67 staining in 231-Control tumors, it failed to decrease the numbers of Ki67-positive cells in 231-MENA and 231-MENAINV tumors (Fig. 2C and D). In contrast, treatment did lead to an increase in cell death as marked by CC3-positive cells in all tumors (Fig. 2E and F). These data indicate that during paclitaxel treatment of tumor-bearing animals, MENA- or MENAINV-expressing tumor cells continue to proliferate but exhibit similar rates of apoptosis to control tumors.

Paclitaxel treatment decreases cell velocity in vitro but does not affect MENAINV-driven tumor cell motility and dissemination in vivo

MENA and MENAINV drive increased cell motility and metastasis during tumor progression (17, 27). Therefore, we examined...
whether MENA isoform expression impacts cell migration and dissemination after paclitaxel treatment. In vitro, paclitaxel treatment decreased velocity of the 3 MENA isoform–expressing cell lines (Supplementary Fig. S2). However, at every concentration of the drug used, 231-MENAINV maintained higher velocity than cells expressing MENA or control cells. Using multiphoton intravital imaging we found that, \textit{in vivo}, paclitaxel treatment significantly reduced the number of cells moving within 231-Control tumors. On the contrary, motility of 231-MENA and of 231-MENAINV tumor cells was not affected by the treatment (Fig. 3A). To investigate the effect of paclitaxel treatment on metastatic burden, we counted the number of colonies in cultured bone

Figure 4.

Paclitaxel treatment selects for high MENA expression \textit{in vitro} and \textit{in vivo}. A, Representative Western blot analysis of whole-cell lysates prepared from multiple breast cancer cell lines treated with 100 nmol/L paclitaxel or DMSO as vehicle for 72 hours and probed with anti-MENA and anti-GAPDH antibodies. Images are not all from the same blots. B, Quantification of endogenous MENA levels after 100 nmol/L paclitaxel relative to DMSO-treated. Data presented as mean \pm SEM for 3 independent experiments. C, FACS analysis of GFP expression levels of 231-Control, 231-MENA, and 231-MENAINV cells treated with docetaxel for 72 hours. The number shows the fold change in GFP signal relative to 231-Control cells. D, Representative images of formalin-fixed, paraffin-embedded (FFPE) section from 231-Control tumor grown in mice treated with paclitaxel or with vehicle and stained for 231-MENA (green), 231-MENAINV (red), and DAPI (blue). Scale bar, 200 \( \mu \)m. Mean of MENA (E) and MENAINV (F) fluorescence signal intensity. Data are presented as mean \pm SEM for 10 fields of view per tumor, from 3 different mice. Statistics determined by unpaired \( t \) test. *, \( P < 0.05 \).
morow and the number of metastases in the lung from mice bearing 231-Control, 231-MENA, or 231-MENA\textsuperscript{INV} tumors for 12 weeks. Neither the number of bone marrow colonies (Fig. 3B) nor the number of lung metastases (Fig. 3C and D) from 231-MENA or 231-MENA\textsuperscript{INV} tumors was affected by treatment with paclitaxel. These data suggest that highly metastatic cells, such as those expressing MENA isoforms, are not affected by paclitaxel treatment in the context of metastatic disease.

**Paclitaxel treatment selects for high MENA expression in vitro and in vivo**

Our results so far indicated that increased MENA or MENA\textsuperscript{INV} expression levels are associated with reduced responses to paclitaxel. We next investigated the effect of paclitaxel treatment on levels of MENA expression in cell populations in vitro and in vivo. First, we analyzed endogenous MENA expression by Western blotting in 5 breast cancer cell lines that were exposed to 100 nmol/L of paclitaxel or a vehicle control (Fig. 4A). We found that 72 hours after paclitaxel treatment, some cell lines (MDA-MB-231 and MDA-MB-175VI) showed increased MENA expression (Fig. 4B). We performed a similar analysis using MDA-MB-231 cell populations expressing heterogeneous levels of GFP, GFP-MENA, or GFP-MENA\textsuperscript{INV}. FACS analysis revealed that treatment with docetaxel (a taxane closely related to paclitaxel) selected for cells expressing higher levels of GFP-MENA or GFP-MENA\textsuperscript{INV}, but not of GFP (Fig. 4C). Finally, quantitative immunofluorescent analysis of tissue sections from 231-Control tumors taken from animals that were treated with either paclitaxel or a vehicle control revealed significant increases in total MENA levels, detected by a pan-MENA antibody, and in MENA\textsuperscript{INV} levels, detected by an anti-MENA\textsuperscript{INV} isoform–specific antibody, in tumors from the paclitaxel-treated mice compared with vehicle (Fig. 4D–F). Together, these data indicate that, both in vitro and in vivo, paclitaxel treatment selects for tumor cells expressing a higher level of MENA and MENA\textsuperscript{INV}.

**MENA isoform–driven resistance does not involve drug efflux or focal adhesion signaling but does affect cell division**

We next investigated the mechanism by which MENA and MENA\textsuperscript{INV} increase resistance to paclitaxel. Paclitaxel efflux through the MDR1 pump is one of the most frequent and best described mechanisms of paclitaxel resistance (9). Co-treatment with HM30181, a third-generation MDR1 inhibitor (31), and 100 nmol/L of paclitaxel negligibly affected the fraction of viable 231-Control cells and did not increase paclitaxel efficacy in 231-MENA\textsuperscript{INV} cells (Supplementary Fig. S3A). Focal adhesion signaling has been reported to promote resistance to paclitaxel (36), and antibodies that detect such modifications can regulate their function (37). We measured the relative abundance of stable (Glu-MT) versus dynamic (Tyr-MT) microtubules in individual cells by immunofluorescence with anti-Glu-MT and anti-Tyr-MT antibodies (Fig. 5A and B). In 231-Control cells, treatment with paclitaxel led to a significant increase in the relative ratio of stable to dynamic microtubules. However, in both 231-MENA and 231-MENA\textsuperscript{INV} cells, there was no change in the relative levels of stable to dynamic microtubules. Together, these data demonstrate that MENA isoforms affect microtubule length and that MENA isoform expression maintains dynamic microtubules during paclitaxel treatment.

**Expression of MENA is associated with increased ratio of dynamic to stable microtubules during paclitaxel treatment**

Paclitaxel promotes cell death by increasing the stability of microtubules, and pathways driving increased microtubule dynamics are known to promote resistance to taxanes (10). Therefore, we examined microtubule structure and dynamics in MENA isoform–expressing cells during paclitaxel treatment. We found that at baseline, 231-MENA and 231-MENA\textsuperscript{INV} cells contained longer microtubules (Supplementary Fig. S5A–S5C). Paclitaxel treatment had no effect on microtubule length in either 231-Control or 231-MENA cells but did elicit a small but significant increase in 231-MENA\textsuperscript{INV} cells (Supplementary Fig. S4H). Together, these data suggest that MENA isoform expression confers the ability to progress through cell division more effectively and successfully during treatment with paclitaxel.

**MENA drives resistance to paclitaxel by increasing MAPK signaling**

The MAPK signaling cascade is among the key pathways known to interact with microtubules. Both ERK1/2 interact with microtubules; microtubule stabilization by paclitaxel increases ERK phosphorylation and, in turn, ERK pathway activation increases microtubule dynamics (10). We measured levels of ERK phosphorylation in 231-Control, 231-MENA, and 231-MENA\textsuperscript{INV} cell lines after 72 hours of paclitaxel treatment. We found that 231-MENA and 231-MENA\textsuperscript{INV} cells had higher levels of pERK Y204
MENA expression alters microtubule dynamics during paclitaxel treatment. Representative images of 231-Control (A) and 231-MENA

relative to 231-Control cells, after paclitaxel treatment, whereas total ERK levels were unchanged in the same conditions (Fig. 6A and B and Supplementary Fig. S6A and S6B). In contrast, treatment with paclitaxel decreased pAkt473 levels equally in all 3 cell lines, without significantly changing total Akt levels (Supplementary Fig. S6C–S6E). We therefore asked whether MEK inhibition (MEKi) could make MENA isoform–expressing cells more sensitive to paclitaxel. In all cell lines, we found significant additive effects between paclitaxel and MEKi PD0325901 in a proliferation assay (Fig. 6C–E), where treatment with both drugs simultaneously led to a greater increase in cell death than with each drug alone. However, higher concentrations of each drug were needed in 231-MENA and 231-MENA

Figure 5.

MENA expression alters microtubule dynamics during paclitaxel treatment. Representative images of 231-Control (A) and 231-MENA

Discussion
Previous work has identified several MENA isoforms, in particular MENA

We observed an inverse correlation between the levels of endogenous MENA expression in cultured breast cancer cell lines and sensitivity to paclitaxel. Ectopic expression of MENA or MENAINV in cultured MDA-MB-231 cells, which have low levels of endogenous MENA, decreased sensitivity to paclitaxel. Conversely, in T47D cells, which endogenously express high levels of MENA and MENAINV, depletion of all MENA isoforms increased sensitivity to paclitaxel. Together, these data indicate that MENA expression promotes resistance to paclitaxel. Because MENAINV as well as MENA are expressed in T47D and in some of the other cell lines in our analysis, it is possible that MENAINV1a can contribute to paclitaxel resistance, although we did not perform any experiments to address whether or not MENAINV1a plays a functional role in resistance to paclitaxel. In this context, however, it is of interest to note that, while a role for MENAINV1a in resistance to chemotherapy remains unknown, MENAINV1a expression contributes to resistance to PI3K inhibitors in HER2-overexpressing breast cancer cells (38). While beyond the scope of this study, it will be interesting to determine the extent to which other MENAINV1a and other MENA isoforms may contribute to paclitaxel resistance and how taxane therapy may affect expression of all MENA isoforms in patients.

Superficially, it may seem paradoxical that aggressive breast cancer cell lines, such as MDA-MB-231 and BT-549, express low levels of endogenous MENA and only trace levels of MENAINV when cultured in vitro. Our previous results indicate that MENA and MENAINV expression is upregulated significantly in aggressive tumor cell subpopulations when cultured breast cancer cells are implanted to make orthotopic tumors in immunocompromised mice (22). Therefore, it is likely that growth in the tumor microenvironment triggers changes in gene expression and alternative splicing in xenografted cells that increase the abundance of MENA and MENAINV during tumor progression, similar to what is observed in autochthonous mouse mammary carcinomas and human breast tumors (26). As our goal in this study was to determine investigate how MENA isoform expression might affect
patients with breast cancer with aggressive, potentially metastatic disease, we designed our experiments on the basis of knowledge derived from studies of MENA isoform expression in tumor cells in vivo. To mimic the effect of the tumor microenvironment on MENA isoform expression for analyses in vitro, we engineered MDA-MB-231 cells to express MENA or MENAINV, the 2 isoforms expressed in patients with aggressive, metastatic breast cancer. Our experiments demonstrated that MENA isoforms expressed in metastatic tumors confer resistance to paclitaxel, and, conversely, that paclitaxel treatment results in increased expression of MENA and MENAINV in tumors. Because we found that paclitaxel treatment was less effective in reducing metastatic burden in tumors with elevated MENAINV, it is possible that taxane-based therapy may, in some cases, trigger elevated expression of MENAINV expression that, in turn, both promotes metastasis and decreases the efficacy of the treatment. Studies to investigate this possibility are underway.

How MENA/MENAINV might promote resistance to taxanes? We initially hypothesized that the role of MENA in regulating focal adhesion signaling may be important in this context, given the established links between focal adhesions and microtubules (39), as well as the known abundance of MENA at focal adhesion sites and its direct interaction with the α5-integrin subunit (17, 32). We found, however, that interaction with α5 was not required...
for MENA-dependent increases in taxane resistance (Supplementary Fig. S2). After paclitaxel treatment, MENA-expressing cells showed an increase in the abundance of dynamic microtubule populations, in paclitaxel-treated cells (Fig. 5). Therefore, it will be interesting to understand whether MENA influences microtubules via association with microtubule-binding proteins, through an effect on signaling pathways that regulates microtubule dynamics, or both.

Interestingly, under control conditions, our data show that MENA or MENA<sup>INV</sup> expression increased microtubule length, supporting a role for MENA in regulation of microtubule behavior (Supplementary Fig. S4). Consistent with these findings, siRNA depletion of Enabled (Ena), the sole Drosophila MENA ortholog (18) in Drosophila S2 cells, induced significant changes in microtubule dynamics, suggesting a potentially evolutionarily conserved role for MENA in regulating microtubule dynamics. However, under control conditions, we did not detect any changes in tyrosination at the whole-cell level, which may be due to the fact that whole-cell immunofluorescence is not sensitive enough to detect subtle differences (Fig. 5). It is clear, however, that some actin regulatory proteins can regulate microtubule dynamics. For example, formins, actin nucleating and elongation factors, can also act as positive regulators of microtubule organization and stability (40). For example, complexes containing the activated forms of the formins mDia1 and INF2 along with the scaffolding, and microtubule-binding protein IQGAP1 can increase microtubule stabilization via direct interaction with microtubules (41), and microtubule regulators can also influence formin-dependent actin dynamics (42). Interestingly, a genetic screen in Drosophila identified Ena as a dosage-sensitive modifier of phenotypes associated with ectopic expression of the microtubule + TIP tracking protein CLASP (43). Therefore, future work focused on the interplay between the actin-based cell motility machinery and microtubule regulation using fluorescent reporters for microtubule tip proteins coupled to live imaging may yield additional insight into the acquisition of taxane resistance by metastatic cancer cells.

Paclitaxel resistance driven by MENA isoforms leads to sustained microtubule dynamics that, in turn, leads to increased ERK signaling, at least in vitro (Fig. 6). Disruption of microtubule dynamics can lead to ERK phosphorylation, and MAPK activation can inhibit microtubule stabilization (14, 44, 45). Therefore, a feedback mechanism may act to balance MAPK pathway activity with microtubule dynamics. Our data indicating that combined treatment with paclitaxel and MEKi, but not with either drug individually, leads to increased microtubule stability in MENA<sup>INV</sup> cells, raising the possibility that MENA<sup>INV</sup> alters the balance between MAPK signaling and microtubule dynamics (Fig. 6). In a breast cancer cohort, MENA expression, as assessed by IHC, correlated with pERK and pAkt staining, with a higher number of pERK and pAkt positivity in MENA-positive tumors, irrespective of Her2 status (46). Depletion of all MENA isoforms in the MCF7 Her2-overexpressing line decreased ERK signaling and inhibited EGF/NRG1-mediated effects on cell proliferation (46). These data are consistent with a potential role for MENA in regulating ERK signaling. Alternatively, activation of bypass signaling pathways such as the Akt pathway occurs downstream of integrins in response to paclitaxel treatment, even in the absence of differences in G<sub>2</sub>-M arrest (47). Interestingly, there were no MENA isoform–induced differences in the levels of Akt phosphorylation (Supplementary Fig. S6), which were significantly decreased in all 3 cell lines, during paclitaxel treatment. This finding is also consistent with our in vivo data showing that during paclitaxel treatment, MENA isoform expression selectively increases proliferation, which is relatively more sensitive to MAPK signaling, but not apoptosis, which is relatively more sensitive to Akt signaling. Finally, our data suggest that combined treatment of a taxane with a MEKi could bypass MENA isoform–driven resistance (Fig. 6). Several groups have previously shown that treatment with a MEKi can enhance paclitaxel-driven cell death in vitro and in vivo (48–50). Multiple clinical trials are currently underway in advanced solid tumors, such as melanoma and non–small cell lung cancer, testing combinations of taxanes and the MEK inhibitor trametinib (51).

Our data reveal an interesting relationship between the response of highly metastatic cells to taxanes and the effect of taxanes on highly metastatic cell populations in tumors that could have important clinical implications. First, following paclitaxel treatment, MENA and MENA<sup>INV</sup> protein expression was higher in both in vitro and xenograft tumors, suggesting that residual surviving cells have undergone a selection for increased MENA and MENA<sup>INV</sup> levels (Fig. 4). Second, we found that MENA<sup>INV</sup>–driven tumor cell motility and metastasis are not affected by paclitaxel treatment (Fig. 3). Paclitaxel is widely used as adjuvant therapy to prevent breast tumor relapse and metastasis (52). Our data suggest that paclitaxel may be less effective in treating patients who have primary tumors expressing high levels of MENA<sup>INV</sup>. While here we focused on TNBC, reduction in MENA levels in estrogen receptor–positive breast cancer cells also altered sensitivity to paclitaxel (Fig. 1), raising the possibility that this mechanism may be important in other subtypes. Currently, there are no biomarkers that predict response to taxanes in patients (53). MENA isoforms are being developed as biomarkers in breast cancer to predict metastatic potential and to guide patient treatment (54). We also recently developed a MENA<sup>INV</sup>–specific antibody and used it to demonstrate that metastatic tumors express higher MENA<sup>INV</sup> than nonmetastatic primary tumors and that high MENA<sup>INV</sup> protein levels were significantly associated with poor outcome and recurrence in a breast cancer patient cohort (17, 26). While further work is needed to establish a clear link between MENA<sup>INV</sup> expression and resistance to paclitaxel in patients, it will be interesting to study how paclitaxel increases MENA<sup>INV</sup> expression and whether this may contribute to a more aggressive phenotype in posttreatment residual tumor cell populations.

Disclosure of Potential Conflicts of Interest
F.B. Gertler has ownership interest (including patents) in and is a consultant/advisory board member for MetaStat. No potential conflicts of interest were disclosed by the other authors.

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