Tumor Cell–Driven Extracellular Matrix Remodeling Drives Haptotaxis during Metastatic Progression

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Fibronectin (FN) is a major component of the tumor microenvironment, but its role in promoting metastasis is incompletely understood. Here, we show that FN gradients elicit directional movement of breast cancer cells, in vitro and in vivo. Haptotaxis on FN gradients requires direct interaction between α5β1 integrin and MENA, an actin regulator, and involves increases in focal complex signaling and tumor cell-mediated extracellular matrix (ECM) remodeling. Compared with MENA, higher levels of the prometastatic MENAα5 isoform associate with α5, which enables 3-D haptotaxis of tumor cells toward the high FN concentrations typically present in perivascular space and in the periphery of breast tumor tissue. MENAα5 and FN levels were correlated in two breast cancer cohorts, and high levels of MENAα5 were significantly associated with increased tumor recurrence as well as decreased patient survival. Our results identify a novel tumor cell–intrinsic mechanism that promotes metastasis through ECM remodeling and ECM-guided directional migration.

SIGNIFICANCE: Here, we provide new insight into how tumor cell-ECM interactions generate signals and structures that promote directed tumor cell migration, a critical component of metastasis. Our results identify a tumor cell–intrinsic mechanism driven by the actin regulatory protein MENA that promotes ECM remodeling and haptotaxis along FN gradients. *Cancer Discov; 6(5); 516–31. ©2016 AACR.

See related commentary by Santiago-Medina and Yang, p. 474.

INTRODUCTION

The tissue microenvironment is composed of stromal cells and extracellular matrix (ECM) and is known to contribute to tumor progression (1). This compartment is rich in substrate-bound and soluble cues, and provides both the structure and signals that promote tumor cell proliferation, survival, and invasion (2). The most abundant ECM proteins in mouse metastatic breast tumors are fibronectin (FN) and collagens (3). In patients with breast cancer, collagen organization has high prognostic value (4, 5), and increased FN correlates with disease progression and mortality (6, 7). The context and mechanisms by which tumor cells sense and respond to changes in ECM abundance and architecture during invasion and metastasis, however, remain poorly understood.

Cancer cells can respond to a variety of cues in order to locally invade and metastasize. Growth factor–mediated chemotaxis is known to be important for local invasion and metastasis (8). Far less is known about haptotaxis, a process in which cell migration is guided by gradients of surface-bound molecules, such as ECM. This is particularly relevant to cancer, where the amount of FN within tumors can vary greatly, with high concentrations of FN typically found near blood vessels, tumor periphery, and in metastatic sites (9, 10). Although FN can activate intracellular signaling pathways via integrins (11), the predominant class of surface adhesion receptors, cells can also remodel the ECM, for example, by driving integrin-mediated assembly of soluble FN into fibrils (12). FN is also known to play an important role in collagen fibrillogenesis (13). Bidirectional communication between cells and ECM cues regulates cell behavior as well as the composition and structure of the surrounding ECM.

MENA, a member of the ENA/VASP family of actin filament elongation factors, is upregulated in various cancers and undergoes alternative splicing during breast cancer progression (14). MENA binds to the C-terminal end of the cytoplasmic tail of the integrin α5 via an LERER repeat domain absent from other ENA/VASP proteins (15). In fibroblasts, MENA regulates both outside-in and inside-out signaling at focal complexes (FX) via its interaction with α5 (15). MENAα5, an alternatively spliced isoform containing a 19-amino acid inclusion (encoded by the “INV” exon), is expressed in aggressive tumor cell subpopulations (16). MENAα5 expression promotes metastasis by increasing sensitivity to EGF (17) and the efficiency of matrix degradation, invasion, and intravasation (18). Together, these findings led us to hypothesize that the ECM may also play an important role in MENA/MENAα5-driven metastasis. Using preclinical models and analysis of patient samples, we describe a previously unappreciated mechanism of metastasis, where upregulation of MENA and its invasive isoform endows tumor cells with the ability to migrate up FN gradients and fashion their own pathway toward the bloodstream.

RESULTS

MENA Drives Haptotaxis of Tumor Cells on FN Gradients via Its Interactions with α5β1 and F-actin

Based on the recent finding that MENA interacts with the cytoplasmic tail of one of the main FN receptors, α5, we hypothesized that MENA may be involved in directional...
mRNA migration responses to gradients of FN. Using a recently developed microfluidic device, we studied cells migrating on FN gradients by time-lapse imaging and quantified their forward migration index (FMI) to assess haptotaxis (Fig. 1A; refs. 19, 20). First, MV57 fibroblasts, which lack all three ENA/VASP proteins (MENA, VASP, and EVL; ref. 21), migrated actively in the device; however, they failed to haptotax on the FN gradient. Interestingly, MENA+, but not VASP+, EVL-expressing MV57 cells exhibited a robust haptotactic response and migrated up the FN gradient (Fig. 1B), even though expression of each of the three ENA/VASP proteins had previously reported similar effects on cell speed (ref. 21; Supplementary Fig. S1A).

We next examined MENA-dependent effects on haptotaxis of breast cancer cells. In serum-free conditions, MDAMB231 cells become enriched on the FN-coated undersides of porous filters in transwell assays (22); however, we found that this cell type failed to exhibit directional movement on FN gradients (Fig. 1C). MDAMB231 cell lines stably expressing GFP-tagged MENA or control-GFP construct at levels similar to those generated for MENA was required for haptotaxis on 2-D gradients of FN, but not on 2-D laminin (LN) or vitronectin (VN) gradients (Fig. 1C), without affecting cell speed (Supplementary Fig. S1D). Varying the concentration of either VN or LN affected the speed of MDAMB231 and 231-MENA cells, but failed to elicit significant haptotactic responses at any concentration tested (Supplementary Fig. S1D–S1G).

In 3-D collagen gels with FN gradients, MENA expression also induced a strong haptotactic response (Fig. 1D), independently of velocity (Supplementary Fig. S1E). Although the exact concentration of FN in tumors is unknown, FN is expressed by tumor and stromal cells, and accumulates in the perivascular area via leakage from the bloodstream, where FN levels as high as 400 μg/mL have been observed (24). Due to the heterogeneous levels of FN found in tumors, we studied haptotaxis 3-D collagen gels in response to gradients generated from different source concentrations of FN. In high levels of FN (up to 500 μg/mL), 231-GFP and 231-MENA cells were unable to migrate up the FN gradient and instead migrated away from the FN source, indicating that the pro-haptotactic effect of MENA on FN gradients is concentration-dependent.

The role of integrins in FN haptotaxis, in particular the two major FN-binding integrins, the α5β1 and αvβ3 integrins, remains poorly understood. Underexpression of α5β1 by the function-blocking antibody P1D6, but not of αvβ3 by Cilengitide (25), blocked haptotaxis of 231-MENA cells (FMIs decreased by over 90%; Fig. 1E), indicating that MENA-driven FN haptotaxis requires α5β1 signaling specifically. We tested whether MENA’s ability to bind α5 via its LERER domain was required for MENA to support haptotaxis (Fig. 1F). MDAMB231 cell lines stably expressing GFP-tagged MENA in which the LERER domain was deleted to abrogate the interaction between MENA and α5 (231-MENA ΔLERER; ref. 15) showed no apparent defects in protein localization (as judged by the GFP-tag), cell morphology, cell area, or proliferation on plastic at steady state (Supplementary Fig. S1B, S1C, S1F, and S1G). 231-MENA ΔLERER cells failed to haptotax in 3-D to FN (FMIs reduced by over 90%; Fig. 1G); however, their migration velocity was similar to cells expressing intact MENA (Fig. 1H). Similar results were obtained in MV57 fibroblasts on a 2-D FN gradient (Supplementary Fig. S1H and S1I). Previously, we found that, although the LERER domain was required for fibroblast spreading on FN, the F-actin–binding (FAB) site in MENA was dispensable (ref. 15; Fig. 1F). Therefore, we investigated the role of the FAB site of MENA in FN-driven haptotaxis. 231-MENAFAB cells failed to haptotax in an FN gradient in a 3-D collagen gel (Fig. 1G), while also displaying slight reductions in cell velocity (Fig. 1H). Overall, these data demonstrate that sensing changes in FN concentrations depend on α5β1 function, as well as the ability of MENA to bind α5 and to F-actin.

### MENAINV Drives Haptotaxis in High FN Concentrations In Vitro and In Vivo

We next investigated the role of the MENAINV isoform in driving haptotaxis. Cultured cells show little to no detectable MENAINV compared with spontaneous or xenograft mammary tumors (26). MDAMB231 and SUM159 cell lines stably expressing GFP-tagged MENAINV or MENAINVΔLERER constructs at levels similar to those generated for MENA (referred to as 231- or 159-MENAINV or MENAINVΔLERER) were made (Supplementary Fig. S1B and S1C). Surprisingly, unlike MENA, MENAINV enabled tumor cell haptotaxis through gradients of high FN concentrations, even up to 500 μg/mL (Fig. 2A), effects that were independent of velocity (Supplementary Fig. S2A and S2B). In contrast, varied concentration gradients of either VN or LN failed to elicit significant haptotactic responses by 231-MENAINV cells, suggesting this response was specific to FN (Supplementary Fig. S2D and S2E). Inhibition of α5β1, but not of αvβ3, blocked haptotaxis of 231-MENAINV cells (Fig. 2B; Supplementary Fig. S2C). MENAINV also binds the cytoplasmic tail of α5 directly via its LERER domain; we found that 231-MENAINVΔLERER and 231-MENAINVΔFAB cells also failed to haptotax in 3-D to FN (Fig. 2B). These findings were confirmed in 2-D and 3-D collagen gels using SUM159 cells expressing the different GFP-tagged MENA isoforms (Supplementary Fig. S2F–S2I). Overall, these data suggest that expression of MENAINV enables cells to haptotax at higher FN concentrations than MENA.

Although upregulation of FN in aggressive tumors is thought to promote invasion and metastasis, whether FN gradients play a role in guiding tumor cell migration in vivo has not been established. Xenograft tumors were generated in the mammary fat pad of immunocompromised mice using MDAMB231 and SUM159 cells. We assayed the ability of cells from the primary tumor to invade actively into microneedles loaded with collagen and increasing concentrations of FN (27). 231-Control tumor cells were not attracted to FN in vivo (Fig. 2C), whereas 231-MENA tumor cells exhibited a biphasic response with robust invasion by 231-MENA cells at intermediate FN concentrations, but little to no invasion into needles with either low or high FN concentrations (Fig. 2C). Interestingly, 231-MENAINV cells were still attracted into the needles containing the high concentrations of FN (Fig. 2C). Although MENA can promote invasion in vivo in response to intermediate FN gradients, MENAINV allows...
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Figure 1. MENA-driven haptotaxis in vitro on FN gradients in 2-D and 3-D is dependent upon its direct interaction with α5 integrin and F-actin. A, schematic diagram of a microfluidic device used for 2-D or 3-D haptotaxis, representative image of an FN gradient in a collagen gel, and a diagram describing the FMI used to quantify haptotaxis. B, expression of MENA, but not VASP or EVL, in MV D7 fibroblasts drives haptotaxis on a 2-D FN gradient (125 μg/mL at top of gradient), as measured by the FMI. C, 231-MENA cells haptotax when plated on a 2-D FN gradient (125 μg/mL at top of gradient), but not on an LN or VN gradient, as measured by the FMI. D, MDAMB231 and 231-MENA cells plated in a 3-D collagen gel and subjected to increasing concentrations of FN at the top of the gradient. E, inhibition of α5β1 with P1D6 (0.5 μg/mL) blocked MENA-driven haptotaxis in 3-D collagen gels, as measured by FMI, whereas inhibition of αvβ3 with Cilengitide (1 μM) had no effect. F, diagram of structure of MENA and its domains, including the LERER domain and the FAB. Deletion of the LERER domain abrogates the interaction of MENA/MENA INV with α5. G, 231-MENA/LERER and MENA/FAB cells did not haptotax in 3-D collagen gels, and this effect was independent of an effect on velocity (μm/min). H, For each experiment, n = 3 experiments, at least 80 cells tracked per condition. Results show mean ± SEM, significance by one-way ANOVA; *, P < 0.5; **, P < 0.01; and ***, P < 0.005. See Supplementary Fig. S1.
Figure 2. MENAINV drives haptotaxis at high FN concentrations in vivo and in vitro. A, 231-MENAINV cells plated in a 3-D collagen gel and subjected to increasing concentrations of FN at the top of the gradient. B, inhibition of α5 with P1D6 (0.5 μg/mL) blocked MENAINV-driven 3-D haptotaxis, as measured by FMI, whereas inhibition of αvβ3 with Cilengitide had no effect. 231-MENAΔLERER or 231-MENAΔFAB did not haptotax in 3-D (n = 3 experiments, ≥150 cells tracked per condition). C, in vivo invasion assay into needles inserted in tumors generated in NOD/SCID mice with MDAMB231 cells expressing Control-GFP, MENA, or MENAINV. Needles contained 0.5 mg/mL collagen and increasing amounts of FN (n = 4 mice per condition). Results show mean ± SEM. Stars above columns represent significance relative to collagen only by one-way ANOVA. D, representative image of an FN gradient (rhodamine-labeled FN, shown in red) on collagen fibers (shown in white) generated using a microscale implantable device implanted into the tumor (tumor cells labeled with GFP shown in green) and visualized by intravital imaging. Scale bar, 100 μm. FMI of (E) MDAMB231 and (F) SUM159 tumor cells expressing different MENA isoforms in the absence of a device, or in the presence of a device releasing fluorescently labeled FN or similarly sized Dextran (data pooled ≥8 movies from ≥2 mice per condition). Results show mean ± SEM; significance by one-way ANOVA; *, P < 0.5; **, P < 0.01; ***, P < 0.005. See Supplementary Fig. S2.
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patients with high numbers of TMEM (a structure containing FN-loaded devices, 231-MENA INV, but not MENA, in human MDAMB231 and SUM159 cells significantly increased the number of cells moving in the xenograft tumors, an effect dependent on the interaction with α5β1 (Supplementary Fig. S2L and S2M). In tumors implanted with FN-loaded devices, 231-MENAINV, but not 231-Control or 231-MENA, cells moved toward the FN gradient (Fig. 2E, representative images with cell tracks; Supplementary Fig. S2K and Supplementary Videos S1 and S2). In contrast, 231-MENAALERER and MENAINVΔLERER cells did not. Similarly, cells in 159-MENAINV tumors migrated toward the FN, an effect that was absent in 159-Control or 159-MENAΔΔLERER tumor cells (Fig. 2F). Altogether, using the needle collection assay and intravital imaging, we show for the first time that haptotaxis toward FN occurring in vivo is driven by MENAINV and its interaction with α5.

MENA Isoform Expression Correlates with FN and Integrin α5 Expression Levels as Well as Outcome in Patients with Breast Cancer

Previous work demonstrated that forced expression of MENAINV drives metastasis in xenograft tumor models (17) and that MENAINV mRNA levels, as detected by qPCR, are relatively higher in cells that intravasate efficiently and in patients with high numbers of TMEM (a structure containing a tumor cell, macrophage, and endothelial cell associated with the likelihood of metastasis in patients with ER+/HER2+ breast cancer; ref. 29). However, the relationship between MENAINV mRNA or protein levels and patient outcome in patients with breast cancer has not been investigated. First, we analyzed the 1,060 patients with breast cancer in The Cancer Genome Atlas (TCGA) cohort with RNA sequencing (RNA-seq) and clinical data available (30). Because the INV exon was not annotated when the RNA-seq data was first analyzed, we accessed the raw sequence data and mapped reads in each sample to all MENA exons. Separating patients into quartiles according to MENA expression failed to reveal any significant correlations between MENA levels (judged by levels of constitutively included exons) and overall survival in the entire TCGA breast cancer cohort (Supplementary Fig. S3A) or in the subset of patients with >10-year follow-up (Fig. 3A). However, patients with high levels (top 1/4) of MENAINV mRNA (as assessed by the abundance of INV exon sequence reads) exhibited significantly reduced survival compared with patients in each of the three lower quartiles of MENAINV expression (Fig. 3B; Supplementary Fig. S3B). Similar results were found in the node-negative patient subgroup (Supplementary Fig. S3E). Furthermore, both Cox and logistic regression demonstrated that MENAINV was a substantially stronger predictor of poor outcome in patients with 10-year follow-up than MENA alone (Fig. 3C and D; Supplementary Fig. S3C and S3D); models combining MENAINV and MENA expression levels failed to increase the predictive power beyond that of MENAINV. We next studied how MENAINV levels correlated with FN and α5 expression in this dataset. Overall, MENA and MENAINV expression were both significantly correlated with FN, and to a lesser degree α5 (Supplementary Fig. S3F). In particular, in patients with >10-year follow-up, we observed a highly significant correlation between MENA, MENAINV, and FN or α5 in patients that succumbed to their disease, which was absent in surviving patients (Supplementary Fig. S3G and S3H).

Using a newly developed antibody specific for MENAINV (26), we then investigated the relationship between endogenous MENAINV, α5, and FN protein using immunostaining in the MMTV-PyMT spontaneous mouse model of breast cancer (31) and in a previously characterized tissue microarray (TMA) of 300 patients (32). Both MENA and MENAINV are expressed in PyMT tumors (Fig. 3E) and can be detected in cells that also express α5β1 (Fig. 3F). MENAINV expression and distribution significantly correlated with that of FN in this model (Fig. 3G and H). We also found a significant correlation between FN and MENAINV levels in the TMA (Fig. 3I and J). Similarly, in patients represented by the TMA, higher MENAINV levels were significantly correlated with poor outcome (Supplementary Fig. S3J). In addition, patients with recurrent disease, either local or at a distant site, had significantly higher levels of MENAINV (Fig. 3K). Logistic regression analysis indicated that MENAINV expression was a significant predictor of recurrence (coefficient of 0.377; P = 0.0186). An average 4.6-fold increase in MENAINV expression correlated with a 2-fold increase in the number of patients with recurrence (Supplementary Fig. S3J and S3K). Further increases in MENAINV expression did not correlate with further increases in recurrence, suggesting that even small increases in MENAINV protein expression can affect recurrence. Although mRNA levels of either MENAINV or FN alone did not correlate with time to disease recurrence, patients with high levels of both MENAINV and FN showed a statistically significant decrease in time to disease recurrence (Fig. 3L). Together, these data provide the first evidence that MENAINV RNA and protein levels correlate with tumor recurrence and survival and support a link between endogenous MENAINV, α5, and FN expression in patients with breast cancer.

To evaluate the role of MENA/MENAINV-driven haptotaxis in metastasis, we quantified the number of spontaneous metastases in the lungs of mice with MDAMB231 and SUM159 xenografts. Mice were sacrificed when primary tumor size reached 1 cm in diameter. Interestingly, the initial growth rate of 231- and 159-MENAINVΔLERER tumors was slower than that of tumors generated from the other cell lines (Supplementary Fig. S4A and S4B). Although there were only minor changes in proliferation levels as detected using the marker Ki67, 231-MENAINVΔLERER tumors had a 6-fold increase in levels of the apoptotic marker cleaved caspase 3 (Supplementary Fig. S4D and S4E). This delay in tumor growth and increase in cell death could be rescued by coinjecting 231-MENAINVΔLERER cells with wild-type MDAMB231 cells expressing mCherry or by letting them grow for 12 weeks instead of 8 to reach 1 cm in diameter (Supplementary Fig. S4D and S4E). Expression of MENAINV

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**Figure 3.** MENAINV is associated with poor outcome in human tumors and requires its interaction with α5 integrin for metastasis. Kaplan-Meier curves for survival of patients with breast cancer categorized by quartiles of MENA (A) or MENAINV (B) mRNA levels, as indicated (Q1 had the highest expression, Q4 the lowest). Data are from 1,288 breast cancer cases with >10 years of follow-up BRCA TCGA dataset (data from entire 1,050 patient cohort in Supplementary Fig. S3A–S3D). Significance calculated by log-rank Mantel–Cox test, HR calculated by log-rank test. Praw calculated by log-rank test for trend (see METHODS). 

**C.** Cox regression carried out to assess the relationship between MENA or MENAINV and time to death in patients with breast cancer (patients with 10-year follow-up). **D.** Logistic regression carried out to assess the relationship between MENA or MENAINV and recurrence; data show mean ± SEM. **E.** Representative images of PyMT-MMTV tumors stained for MENA (red) and MENAINV (green). Scale bar, 20 μm. **F.** Representative images of PyMT-MMTV tumors stained for MENAINV (green) and integrin α5 (red). Same scale as **E.** **G.** Representative image from a wild-type PyMT tumor FN (red), MENAINV (green), and nuclei (DAPI) staining. Scale bar, 100 μm. **H.** Correlation between MENAINV and collagen FN intensity. Data from over 50 fields from 4 PyMT mice, each dot represents an individual field. **I.** Representative image of tumor spot from a TMA with high levels of MENAINV (green) and FN (red). **J.** Correlation between FN and MENAINV staining in the entire patient cohort. MENAINV expression in 300 patients with breast cancer comparing patients with or without recurrence; data show mean ± SEM. **K.** Table showing the median recurrence-free time in months and corresponding P value in patients with high vs. low MENA, high vs. low MENAINV, or high vs. low MENA or FN. Significance calculated by log-rank Mantel–Cox test. **M.** Representative fluorescence images of GFP-positive metastasis in lungs of mice with 231-Control, MENA, or MENAINV tumors. Scale bar, 50 μm. **N.** Hematoxylin and eosin (H&E) images of formalin-fixed, paraffin-embedded sections cut from the lungs of mice bearing MDA MB231 tumors expressing different MENA isoforms. Scale bar, 100 μm. **O.** Lung metastatic index of NOD/SCID mice bearing tumors grown from MDA MB231 cells expressing different GFP-tagged MENA isoforms and measuring at least 1 cm in diameter (n=4 mice per cell line). Data show mean ± SEM, significance by one-way ANOVA, *P* < 0.05; **, P < 0.01; ***, P < 0.005. See Supplementary Figs. S3 and S4.
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significantly increased the metastatic index in the lungs 5-fold in both human cell lines at 8 weeks, compared with control 231s (Fig. 3M–O). Deletion of the LERER region in both MENA and MENAINV significantly decreased the lung metastatic index (Fig. 3N and O). Similar results were found in the poorly metastatic SUM159 cell line (Supplementary Fig. S4E). Similarly, depletion of all MENA isoforms in MDAMB231 cells using shRNA also delayed tumor growth, as well as decreasing tumor cell motility and metastasis (Supplementary Fig. S4G–S4M). These data support a model whereby MENA/MENAINV-driven α5-dependent haptotaxis plays a key role in promoting tumor metastasis.

Haptotaxis on FN Gradients Is Dependent on Signaling at FXs

We next investigated the mechanism by which MENA isoforms drive haptotaxis at low and high FN concentrations. One way FN can support cell migration is by activating integrin-mediated intracellular signaling pathways at FXs that promote cell motility (11). When plated on 2-D collagen and FN, both 231-MENA and 231-MENAINV cells showed increases in cell area and in the number of α5-positive adhesions relative to control cells, or to cells plated on collagen only, an effect dependent on α5 activity as well as the α5-binding LERER domain (Fig. 4A–E). Although 231-MENAINV cells showed a 30% increase in both total and surface levels of α5, the steady-state levels of αv, β1, and other integrins examined were unchanged (Supplementary Fig. S5A–S5G). Furthermore, plating cells on FN also increased the levels of α5 within MENA-positive adhesions (indicated by the GFP tag), with 231-MENAINV cells showing increased levels of α5 relative to 231-MENA cells (Fig. 4B and F). The FN-driven recruitment of α5 to MENA-positive adhesions was dependent on α5 activity as well as the LERER domain for both isoforms (Fig. 4F and G). 231-MENAINVΔLERER cells also had a reduced number of MENA-positive adhesions (Supplementary Fig. S5I).

Phosphorylation of several proteins at focal adhesions downstream of integrin activation, particularly focal adhesion kinase (FAK), is important for motility and tumorigenesis (11, 33). Pharmacologic inhibition of FAK inhibited MENA and MENAINV-driven haptotaxis on low FN gradients (Fig. 4H). Immunofluorescence of haptotaxing cells revealed that both 231-MENA and MENAINV cells show an increased number of pFAK397-positive adhesions (Fig. 4I and J). In 231-MENAINVΔLERER or 231-MENAINVΔFAB cells, the number of pFAK397-positive adhesions in the cells was significantly lower than in 231-MENAINV cells (Fig. 4K). When cells were plated on high FN gradients, only 231-MENAINV cells haptotaxed (Supplementary Fig. S5J), and accordingly, these cells had a significant increase in the number of pFAK397-positive adhesions relative to 231-MENA or control cells (Fig. 4L). These data suggest that outside-in activation of integrins and MENA-dependent FX signaling by FN is important for haptotaxis of tumor cells.

The differences between FX composition, signaling, and haptotaxis arising from expression of MENAINV versus MENA prompted us to ask whether we could detect any relevant biochemical differences between the isoforms. Previously, we demonstrated that MENA could be detected in complex with α5 by coimmunoprecipitation (IP; ref. 15). We performed α5 IPs to compare the amounts of MENA and MENAINV in complex with α5, and reproducibly detected an average 2.2-fold greater level of MENAINV compared with MENA by Western blot of anti-α5 IPs (Fig. 4M and N). Thus, inclusion of INV in MENA leads to significantly increased association with α5β1.

MENAINV-Driven ECM Remodeling Is Required for 3-D Haptotaxis

FN could also support haptotaxis through its role in providing structural support for cells, particularly in 3-D and in the presence of collagen. FN fibrillogenesis exposes cryptic binding sites in FN normally hidden in the globular form (12), and fibrillar FN can bind collagen and regulate its deposition and organization (13). 231-MENA and MENAINV cells had an increased number of protrusions in low FN gradients, whereas in high FN gradients, only 231-MENAINV cells showed this phenotype (Supplementary Fig. S6A). Similarly, 231-MENAINV cells were more elongated than MENA or Control cells in the presence of an FN gradient in vivo, an effect dependent on the interaction with α5 (Supplementary Fig. S6B and S6C). We also found that 231-MENAINV exhibited increased accumulation (20%) of FN (Fig. 5A and B) and collagen (Fig. 5A and C) at both low and high FN concentrations after 24 hours, whereas neither 231-MENA nor 231-Control cells exhibited any significant accumulation of the two ECM proteins. FN-triggered outside-in signaling was insufficient to support MENAINV-dependent haptotaxis in 3-D gels, as 231-MENAINV cells failed to haptotax on a gradient of FN7-11, a fragment of FN that activates signaling via RGD-binding integrins, but cannot form fibrils (ref. 12; Fig. 5D). Inhibition of fibrillogenesis by the addition of a 70 kD FN fragment that contains the cryptic FN- and collagen-binding sites in FN (34) blocked MENAINV-driven 3-D haptotaxis (Fig. 5E). Therefore, the formation of FN fibrils is important for FN to evoke directional motility of tumor cells in 3-D. Addition of the 70 kD FN fragment to spreading 231-MENAINV cells decreased the number of α5-positive adhesions as well as α5 recruitment to MENAINV-positive adhesions (Supplementary Fig. S6D–S6G), suggesting that fibrillar FN may enhance assembly of α5β1-containing adhesions.

In 231-MENAINV cells, inhibition of α5β1 decreased accumulation of both FN and collagen, whereas inhibition of αvβ3 had little or no effect. 231-MENAINVΔLERER and 231-MENAINVΔFAB cells had decreased FN and collagen (Fig. 5F and G) accumulation around the cells. Inhibition of protease activity with the broad-spectrum matrix metalloproteinase inhibitor BB94 had no effect on ECM reorganization, but inhibition of acto-myosin contractility by the ROCK inhibitor Y-27632 did decrease collagen and FN accumulation around the cells (Supplementary Fig. S6H and S6I). These results indicate that MENAINV-driven α5-dependent FN and collagen accumulation by the tumor cells themselves is important for haptotaxis.

MENAINV Expression Drives Collagen Reorganization in Tumors

Given the role of MENAINV in driving ECM reorganization in vitro, the importance of FN for collagen fibrillogenesis (13), and recent published work showing that collagen organization can correlate with disease outcome (5), we next examined...
Figure 4. MENAINV drives haptotaxis via increased FX signaling. A, representative images of 231-Control, MENA, and MENAINV cells plated on FN and collagen, stained with antibodies to α5 and GFP to visualize tagged GFP-MENA or GFP-MENAINV and with phalloidin to visualize F-actin. Scale bar, 5 μm. B, magnification of inset shown in A. Scale bar, 1 μm. C, quantification of cell area (in μm²) of MDAMB231 cells expressing different isoforms when plated on collagen (0.1 mg/mL) + FN (50 μg/mL). D, number of α5-positive adhesions relative to cell area for 231-MENA and 231-MENAINV cells for cells plated on collagen only, collagen (0.1 mg/mL) + FN (50 μg/mL), and in the presence of P1D6, α5-function blocking antibody. E, number of α5-positive adhesions relative to cell area for cells plated on collagen + FN. F, intensity of α5 signal in MENA-positive adhesions (as counted by GFP positivity) for 231-MENA and 231-MENAINV cells plated on collagen only, collagen (0.1 mg/mL) + FN (50 μg/mL), and in the presence of P1D6. α5-function blocking antibody. G, intensity of α5 signal in MENA-positive adhesions (as counted by GFP positivity) in cells plated on a low 2-D 125 μg/mL FN gradient. H, inhibition of FAK (0.5 μmol/L) decreased MENAINV-driven 2-D haptotaxis on a low 2-D 125 μg/mL FN gradient. Data from ≥3 experiments, with ≥80 cells tracked per condition. I, representative images of 231-Control, MENA and MENAINV, 231-MENALERER, and MENAxFAB cells plated on a low 2-D 125 μg/mL FN gradient, stained with pFAK397 (red) and Phalloidin to visualize F-actin (blue). Scale bar, 5 μm. J, quantification of pFAK Y397-positive adhesions in 231-Control, MENA, or MENAINV, while plated on a 2-D low 125 μg/mL FN gradient. K, 231-MENALERER or 231-MENAINV/FAB cells had decreased number of pFAK Y397-positive adhesions on a 2-D low 125 μg/mL FN gradient. L, quantification of pFAK Y397-positive adhesions in 231-Control, MENA, or MENAINV, while plated on a 2-D high 500 μg/mL FN gradient. Data pooled from 3 experiments, with at least 30 cells analyzed per condition. M, representative image of a WB for α5 immunoprecipitation from 231-MENA and 231-MENAINV lysates, probed for MENA, α5, and Tubulin. N, quantification of fold increase in MENA pulled down in α5-IP, n = 4. For all staining experiments, data pooled from at least three experiments, with at least 10 cells analyzed per experiments. Results show mean ± SEM, significance by one-way ANOVA; *, P < 0.5; **, P < 0.01; ***, P < 0.005. Stars above data set represent significance relative to control. See Supplementary Fig. S5.
Figure 5. MENAINV-dependent directional motility requires ECM reorganization in vitro. A, representative images of MDAMB231 cells (outlined in white) and 231-MENAINV cells (green) in a 3-D 125 μg/mL FN gradient (red) in a collagen gel (blue) in merged image; middle and right plots show grayscale images of FN and collagen alone, respectively. Scale bar, 25 μm. 231-MENAINV showed increased accumulation and reorganization of (B) FN and (C) collagen, in both low 125 μg/mL and high 500 μg/mL 3-D FN gradients. D, a gradient of recombinant 7-11 domains of FN failed to induce 3-D haptotaxis of 231-MENAINV cells, as measured by the FMI. E, inhibition of FN fibrillogenesis by inclusion of a 70 kD fragment ablated MENAINV-dependent haptotaxis in 3-D FN gradients (n = 3 experiments, ≥80 cells tracked per condition). Deletion of the FAB and LERER regions in MENAINV, and inhibition of α5β1 with P1D6, but not Cilengitide, reduced collagen (F) and collagen (G) accumulation at low FN concentrations. Data from ≥3 experiments, with ≥30 cells analyzed per condition. Results show mean ± SEM, significance by one-way ANOVA; *, P < 0.5; **, P < 0.01; ***, P < 0.005. See Supplementary Fig. S6.
whether the changes in ECM accumulation observed short-term in vitro translated into significant changes in the structure and abundance of collagen in vivo. Using Second Harmonic Generation (SHG), we examined the structure and abundance of collagen in multiple tumors to investigate whether MENA and MENA$^{INV}$ were associated with changes in collagen abundance and organization in vivo. We examined the thickness of the collagen capsule surrounding the tumors. Intravital imaging of tumors revealed that deletion of the LERER in both 231-MENA and MENA$^{INV}$ tumors leads to significantly thicker collagen capsules (Supplementary Fig. S6). Similarly, knockdown of MENA in 231 cells also led to an increase in collagen thickness (Supplementary Fig. S6K). Histologic analysis of tumor sections showed that tumors in MMTV-PyMT mice null for MENA, known to have significantly lower levels of metastasis (35), showed significantly increased collagen levels compared with wild-type mice (Fig. 6A and B). Endogenous MENA$^{INV}$ levels in wild-type PyMT tumors were correlated
with lower levels of collagen (Fig. 6C and D). In patients with high MENAINV expression, there was significantly lower collagen signal than in patients with low MENAINV expression (Fig. 6E and F). Together, these data suggest that highly invasive tumors arising from, or correlating with, MENAINV expression were associated with thinner collagen capsules, whereas the poorly metastatic tumors arising from MENA-deficient cells were associated with thicker collagen capsules.

Although the amount of collagen deposition surrounding the tumor is an important factor in local invasion, changes in collagen shape and orientation have also been linked with invasiveness. A lower density of collagen organized into straight fibers oriented perpendicularly to the edge of the tumor has been associated with poor outcome in a breast cancer cohort (5). Representative images of the collagen at the edge of the MDAmb231 and SUM159 xenograft tumors show 231-Control and 231-MENA tumors had dense, curly collagen fibers, organized in all directions relative to the tumor edge (Fig. 6G and H; Supplementary Fig. S6L). However, 231-MENA tumors displayed a significantly increased frequency of fibers oriented perpendicular to the tumor edge (Fig. 6G and H). 231-MENALERER and 231-MENAΔLERER tumors had collagen fibers parallel to the tumor edge (Supplementary Fig. S6L and S6M). Indeed, these tumors contained abundant collagen fibers with very small angles relative to the tumor edge. Both phenotypes were also observed in tumors generated with SUM159 cells (Fig. 6G; Supplementary Fig. S6N and S6O).

Together, these results indicate that the initial MENAINV-dependent changes in ECM accumulation observed in our short-term in vitro assays can translate in vivo (over much longer time scales associated with tumor progression) into significant reductions in overall accumulation of encapsulating collagen accompanied by increased abundance of linear fibers oriented perpendicularly to the tumor margin.

**DISCUSSION**

Our results lead us to propose a novel mechanism by which tumor cells sense, respond to, and reorganize the ECM to support metastasis (summarized in Fig. 7). Integrin-dependent outside-in signaling at FXs, as well as inside-out ECM remodeling, is necessary for directional migration toward FN. FN and α5 expression are upregulated in breast cancer tumors, and high levels of tumor FN and α5 have been associated with poor outcome (7), though not, by themselves, in the datasets we analyzed. Given the heterogeneous levels of FN within both the primary tumor and metastatic niche, disseminating cells must be able to migrate effectively through areas of different FN concentrations, as well as to move from areas of low FN to areas of high FN (and vice versa). In addition to simply being permissive for migration, we show that FN acts as a directional cue in vivo. Expression of MENA drives haptotaxis at low FN concentrations, whereas expression of MENANINV allows cells to migrate at high concentrations of FN similar to those present around blood vessels in tumors (9). Abrogation of the interaction between MENA/MENAINV and α5β1 abolished haptotaxis in vitro while also significantly decreasing metastasis in two mouse xenograft breast cancer models. Together, these findings support the idea that FN can act as a potent guidance and motility cue for tumor cells during metastatic progression.

At a mechanistic level, inclusion of the INV sequence increases the association of MENA with α5 by 2.2-fold. We propose that increased association of MENAINV versus MENA with α5 likely underlies the isoform-specific differences in α5β1-mediated effects on haptotaxis and FXs. The INV sequence is inserted between the amino terminal EVH1 domain in MENA, which mediates interactions with several other molecules associated with integrin function, and the LERER domain, which binds directly to α5 (15, 36). It will be of great interest to determine exactly how the INV sequence modifies interactions with α5.

The ECM can also deliver signals to other types of receptors by providing binding sites for their ligands (37). Although it is well established that proteoglycans directly bind growth factors, allowing the ECM to act as a reservoir for invasive signals (38), emerging evidence suggests that ECM proteins contain domains that may allow them to activate receptor tyrosine kinases directly. FN-mediated activation of α5β1 can, for example, lead to hepatocyte growth factor (HGF)–independent activation of MET to promote invasion in ovarian cancer cells (39). In addition, expression of mutant p53 protein, which is mutated in 50% of cancers (40), can drive invasion through enhanced
RCP-dependent corecycling of α5β1 with several receptor tyrosine kinases (RTK), including EGFR and MET (25, 41). Given that MENAINV expression sensitizes cells to EGF, enabling them to invade and migrate in response to low EGF concentrations (17, 18, 42), it will be interesting to investigate the potential role of cross-talk with RTK signaling in MENAINV-driven haptotaxis.

The importance of tumor cell–driven FN fibrillogenesis and ECM remodeling in MENAINV-driven haptotaxis is surprising. FN polymerization is required for deposition of collagen I (34), and antibody binding to the collagen-binding site on FN inhibits collagen fibrillogenesis (43). Here, we show for the first time that tumor cell–mediated FN fibrillogenesis is required for haptotaxis, a process driven by expression of MENAINV. We also show the first evidence for tumor cell–driven collagen reorganization in vivo, also driven by MENAINV and its interaction with α5β1. Together, these data suggest that tumor cells can pave their own way to blood vessels. Although the mechanism by which FN and integrins drive collagen fibrillogenesis remains incompletely understood, our results indicate that MENAINV provides a link between integrin activation and the cytoskeleton to drive ECM organization by tumor cells. The MENAINV-driven reduction in collagen encapsulation and increase in linear fibrils radiating from the tumor periphery, in turn, further enhance metastatic phenotypes. Overall, our findings highlight the potential importance of bidirectional integrin-mediated signaling in the tumor cell compartment in addition to the previously characterized contributions from stromal cells (4) in the regulation of ECM structure.

Our data also support a role for the relationship between MENAINV, α5, and FN in human breast cancer. Using an isoform-specific antibody and bioinformatic analysis of available TCGA data, we found that high expression levels of MENAINV and FN are associated with increased recurrence and poor outcome in two human breast cancer cohorts. Future studies on larger patient cohorts will be needed to help determine the utility of MENAINV as a diagnostic and prognostic marker, and whether it provides additional information when used in conjunction with other tumor markers. Altogether, our findings reinforce the importance of MENAINV in human breast cancer and suggest that targeting the chemotactic and haptotactic pathways by which MENAINV promotes invasion could be useful therapeutically for metastatic breast cancer.

METHODS

Antibody Reagents, Growth Factors, and Inhibitors

Antibodies used in the study are as follows: α5 (for immunofluorescence: BD Biosciences, #555651 at 1:1,500; for iP: Millipore, AB1928; and for Western Blot: Santa Cruz Biotechnology, sc-166681 at 1:1,000), αv (BD Biosciences; 611012 at 1:500), α6 (Abcam; ab105566 at 1:1,000), α2 (Abcam; ab133557), β1 (BD Biosciences; 610467 at 1:500), FAK (BD Biosciences; 610087), pFAK Y397 (Invitrogen; 44-625G at 1:300), cleaved caspase 3 (Cell Signaling Technology; 9661 at 1:300), Ki67 (Cell Signaling Technology; 9027 at 1:300), FN (gift from R.O. Hynes, at 1:1,000). See ref. 26 for description of MENAINV–rabbit monoclonal antibody. Animals were immunized with a peptide containing the sequence encoded by the INV exon. Clones were screened for MENAINV specificity in Western blot assays and by immunostaining of formalin-fixed, paraffin-embedded tumor sections from wild-type or MENA-null mice (Supplementary Fig. S3; ref. 26). Cilengitide (Selleck Chemicals), P1D6 α5 blocking antibody (DSHB), FAKi (Santa Cruz Biotechnology), 70 kD fragment, and its control peptide for blockade of fibrillogenesis (gift from Dr. Sottile, University of Rochester), FN 7-11, purified from a plasmaid from R.O. Hynes.

Cell Culture

MDAMB231 cells were purchased directly from the ATCC in June 2012, where cell lines are authenticated by short tandem repeat profiling. These cells were not reauthenticated by our lab and were cultured in DMEM with 10% FBS (HyClone). SUM159 cells were obtained from Joan Brugge’s lab at Harvard Medical School (January 2011) and were not reauthenticatced in our lab. SUM159 cells were cultured according to the ATCC protocols. MV07 fibroblasts were isolated from mice in our lab in October 1999 and cultured as previously described (15). These cell lines were authenticated in our lab, and deletion of MENA was verified at the mRNA and protein level. Retroviral packaging, infection, and FACS were performed as previously described (15). Cell lines were engineered to express MENA isoforms stably at 10- to 15-fold higher levels than parental lines. MV07 fibroblasts isolated were maintained at 32°C, 5% CO2 in DMEM supplemented with 1-Glutamine, penicillin, and streptomycin, 15% fetal bovine serum, and 50 U/mL interferon (4777; Sigma; ref. 15). Stable knockdown cell lines were generated using mir-30–based shRNA sequence “CAGAAGACATCNGCCCTTAA” for MENA expressing an mCherry tag.

Immunohistochemistry

Fixation, processing, and staining of tissue sections from tumors were carried out as previously described (18). 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 endogenous peroxidase inactivation, sections were incubated with primary antibodies overnight at 4°C and fluorescently labeled secondary antibodies at room temperature for 2 hours. Sections were stained using the following antibodies: anti-MENA (1:500), anti-Ki67 (BD Biosciences), and cleaved caspase 3 (BD Biosciences). Fluorochromes on secondary antibodies included AlexaFluor 594, AlexaFluor488, and AlexaFluor647 (Jackson Immunoresearch). Sections were mounted in Fluoromount mounting media and imaged at room temperature. Z series of images were taken on an Applied Precision DeltaVision microscope using Softworx acquisition, an Olympus 40X1.3 numerical aperture (NA) plan apo objective, and a Photometrics CoolSNAP HQ camera. Images were deconvolved using Deltavision Softworx software and objective specific point spread function. At least four images were captured for each tumor, with at least three tumors per tumor group.

TMA

Details of the patient cohort and associated data used to generate the TMA are published (32). The TMA was stained by immuno-fluorescence and imaged with a Vectra-automated slide scanner and a 20x objective. The field of view with this objective covers 90% of the core spot. Each patient had three cores on the TMA. All were imaged, but some had to removed due to lack of tissue or folded tissue. Fluorescence intensity in the tumor compartment was analyzed using Inform software. MENAINV and FN intensity metrics are in arbitrary units.

Haptotaxis Assays

Microfluidic devices were prepared as described (19). For haptotaxis on a 2-D matrix, after bonding polydimethylsioxane devices to MatTek dishes, the chamber was coated with 0.1 mg/mL Collagen I for 1 hour at 37°C, and then 250 μg/mL fluorescently labeled FN was flowed
through the source channel for 1 hour. Cells were then plated in the device in full serum media and left to attach for 1 hour before imaging. For haptotaxis in a 3-D matrix, cells were resuspended in 1 mg/mL collagen I (BD Biosciences) with 10× DMEM and 1N NaOH and 3 mmol/L EGF in full serum media, plated in the cell culture chamber, and left to settle for 8 hours at 37°C. Fluorescently labeled FN was then flowed through the source channel for 1 hour before imaging. For all haptotaxis experiments, the FN concentration represents the concentration at the top of the gradient. Unless mentioned, FN concentration at the top of the gradient is 125 μg/mL. Cells were imaged overnight in the haptotaxis device, with images being acquired every 10 minutes for 16 hours in an environmentally controlled microscope (TE2000; Nikon) with a 20× objective and a Photometrics Coolsnap HQ camera. Individual cells were manually tracked using ImageJ software Manual Tracking plug-in. The tracks obtained were analyzed using the Chemo-taxis Tool ImageJ plugin (from Ibidi). This analysis tool was used to extract the FMI (Fig. 1A) along with the velocity of migration and the persistence of migration using the D (net path length)/T (total path length) ratio (Fig. 1A; refs. 20, 44).

**Tumor Formation and Metastasis Assay**

All animal experiments were approved by the MIT Division of Comparative Medicine. For xenograft experiments, MDAMB231 or SUM159 cells (2 million per mouse in PBS and 20% Collagen I) expressing different MENA isoforms were injected into the fourth right mammary fat pad of 6-week-old female NOD/SCID mice (Tacomic). Tumor size was measured weekly with calipers. Eight or 12 weeks after surgery, once tumors had reached 1 cm in diameter, mice were used for intravital imaging, and then sacrificed and their tumors and lungs were fixed in 4% formalin overnight. Metastatic index was calculated by counting the number of metastases in each lobe relative to the weight of the tumor. Each tumor group contained 4 to 6 mice. PyMT-MMTV mice were obtained from Jackson, and mice were left to growth for 15 to 20 weeks, when they had developed tumors of about 1.5 cm in diameter. Histologic analysis of hematoxylin and eosin (H&E) from these tumors confirmed these tumors were advanced carcinoma.

**In Vivo Invasion Assay**

The in vivo invasion assay was performed in at least 4 mice per condition as previously described (45). Briefly, needles were held in place by a micromanipulator around a single mammary tumor of an anesthetized mouse. Needles contained a mixture of 0.5 mg/mL Collagen I, EDTA with L-15 media or increasing amounts with FN. After 4 hours, the contents of the needles were extruded. Cells were stained with DAPI and counted.

**Intravital Imaging and In Vivo Haptotaxis**

Intravital multiphoton imaging was performed as described previously (23) using a 25 × 1.05 NA water immersion objective with correction lens. For in vivo haptotaxis, a microscale device filled with multiple reservoirs (28) with powdered rhodamine-FN or dextran was prepared. After exposing the tumor, the device was implanted into the tumor edge. Hour-long time-lapse movies were analyzed for frequency of motility and tracking, and to measure and quantify cell characteristics in 3-D and over time using NIH ImageJ. Cells that were either protruding or moving were counted as motile. For each movie, the FMI was calculated, with the angle for each track made relative to the direction of FN gradient for each frame. Data are pooled from 2 to 4 mice per tumor group, with 4 to 10 fields imaged per mouse. Data were pooled from 2 to 4 mice per tumor group, with 4 to 10 fields imaged per mouse.

**Western Blot/Immunoprecipitation**

For the in vivo immunoprecipitation, 231-MENA and 231-MENAINV were lysed with CSK buffer (10 mmol/L PIPES—pH 6.8, 50 mmol/L NaCl, 150 mmol/L sucrose, 3 mmol/L MgCl2, 1 mmol/L MnCl2, 0.5% Triton X-10, protease and phosphatase inhibitors) and passed through a 23-gauge needle. Lysates were precleared with protein A beads for 1.5 hours, incubated with the integrin α5 antibody (1928; Millipore) for 2.5 hours at 4°C, and then captured with 3% BSA-blocked protein A beads for 2 hours. Beads were washed 3 times in CSK lysis buffer, and proteins were eluted in 2× sample buffer. Standard procedures were used for protein electrophoresis, Western blotting, and immunoprecipitation. MDAMB231 expressing different MENA isoforms were lysed in 25 mmol/L Tris, 150 mmol/L NaCl, 10% glycerol, 1% NP 40, and 0.5 mol/L EDTA with a protease Mini-complete protease inhibitors (Roche) and a phosphatase inhibitor cocktail (PhosSTOP; Roche) at 4°C. Protein lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked with Odyssey Blocking Buffer (LiCor), and incubated in primary antibody overnight at 4°C. Proteins were detected using LiCor secondary antibodies. Protein level intensity was measured with ImageJ, and data were pooled from at least three different experiments.

**FACS**

MDAMB231 cells expressing the different isoforms were trypsinized, resuspended in media, and then incubated with a primary antibody in PBS and 5% media for 30 minutes on ice. Next, the cells were incubated with a species appropriate Alexa647-tagged secondary antibody and then resuspended in PBS with 10 μg/mL propidium iodide. Samples were then analyzed on a FACS-Calibur machine (BD Biosciences). Data are pooled from at least three separate experiments, with 10,000 cells analyzed per experiment.

**Immunofluorescence**

Cells were plated in a haptotaxis device on a 125 μg/mL 2D FN gradient for 3 hours or on collagen-coated glass-bottomed dishes (MatTek) in serum-free media for 30 minutes at 37°C. Cells were then fixed for 20 minutes in 4% paraformaldehyde in PHEM buffer, then permeabilized with 0.2% Triton X-100, blocked with 10% BSA, and incubated with primary antibodies overnight at 4°C. Z series of images were taken on an Applied Precision DeltaVision microscope using Softworx acquisition, an Olympus 40 × 1.3 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 ImageJ. Images are pooled from at least three independent experiments, at least 10 cells per experiment.

**MENA**(INV) TCGA Data Retrieval**

RNA-seq data in fastq format were obtained from TCGA. For each sample, ENAH (MENA)-derived reads were extracted from the full dataset by aligning to a target database that contained collection of all possible ENAH isoforms using BWA version 0.7.10. Properly paired ENAH reads were then extracted withSamtools version 0.1.19. ENAH isoforms were then quantified by aligning to hg19 using tophat2 version 2.0.12 guided with an edited GTF file derived from the UCSC known genes annotation that contained all ENAH variants of interest. Bedtools version 2.20.1 and a custom python script were used to count reads that overlap with each ENAH exon. The resulting counts per exon were then normalized for RNA loading by calculating a counts per million reads per Kb of mRNA using a sum of exon-level counts in the publicly available and preprocessed TCGA data as the total aligned counts denominator.
Survival/Recurrence Data Analysis

The relationship between MENA/MENAINV expression levels (from mRNA TCGA or protein TMA) and survival (time to death) or metastasis (time to recurrence) was assessed by the log-rank Mantel-Cox test. In each sample, patients were divided into quartiles according MENA or MENAINV expression (Q1 being the highest level of expression and Q4 being the lowest). The HR for each quartile (with 95% confidence interval values) was calculated. The P value generated by this log-rank test evaluates whether the difference in the curves is significant. We also performed the log-rank test for trend to further assess the differences between the curves representing patients with varying levels of MENA isoform expression.

The hazard effects of MENAINV and MENA upon the time to death were investigated by Cox regression using R 2.15.3 basing on TCGA strength of the association.

The relationship between MENA/MENAINV expression levels and survival status of TCGA BRCA subjects was evaluated by logistic regression using P values and coefficients corresponding to the independent variables. The hazard effects of MENAINV and MENA upon the time to death of the BRCA patients in the TCGA study. The association between MENAINV/MENA expression level and survival status of TCGA BRCA subjects was evaluated by logistic regression using R 2.15.3. In order to compare coefficients across tests, we first standardized INV and MENA values to mean zero and standard deviation one. Logistic regressions were conducted by choosing survival status as dependent variable (1 as death, and 0 as alive). The only independent variable fitted in the model was INV or MENA, respectively. P values and coefficients corresponding to the independent variables were used to judge the significance of the association as well as the strength of the association.

Disclosure of Potential Conflicts of Interest

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

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Haptotaxis and Metastasis

Tumor Cell–Driven Extracellular Matrix Remodeling Drives Haptotaxis during Metastatic Progression

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