ABSTRACT

Membrane type 1 matrix metalloproteinase (MT1-MMP, MMP-14) is a transmembrane collagenase highly expressed in metastatic ovarian cancer and correlates with poor survival. Accumulating evidence shows that the cytoplasmic tail of MT1-MMP is subjected to phosphorylation, and this post-translational modification regulates enzymatic activity at the cell surface. To investigate the potential role of MT1-MMP cytoplasmic residue Thr\textsuperscript{567} phosphorylation in regulation of metastasis-associated behaviors, ovarian cancer cells that express low endogenous levels of MT1-MMP were engineered to express wild-type MT1-MMP, a phospho-mimetic mutant (T567E) or a phospho-deficient mutant (T567A). Results show that Thr\textsuperscript{567} modulation influences behavior of both individual cells and multi-cellular aggregates (MCAs). The acquisition of either wild-type or mutant MT1-MMP expression results in altered cohesion of epithelial sheets and the formation of more compact MCAs relative to parental cells. Cells expressing MT1-MMP-T567E phospho-mimetic mutants exhibit enhanced cell migration. Furthermore, MCAs formed from MT1-MMP-T567E-expressing cells adhere avidly to both intact ex vivo peritoneal explants and 3-dimensional collagen gels. Interaction of these MCAs with peritoneal mesothelium disrupts mesothelial integrity, exposing the sub-mesothelial collagen matrix on which MT1-MMP-T567E MCAs rapidly disperse. Together, these findings suggest that post-translational regulation of the Thr\textsuperscript{567} in the MT1-MMP cytoplasmic tail may function as a regulatory mechanism to impact ovarian cancer metastatic success.

INTRODUCTION

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancy in the United States (1). Most women (75%) are diagnosed with stage III-IV disease and present at diagnosis with widely disseminated metastasis, resulting in only a ~25% five-year survival rate. However, the survival rates increase to 95% for women diagnosed prior to metastatic dissemination (2). These statistics highlight the urgent need to develop early detection strategies as well as to thoroughly understand the molecular, cellular and biological events that promote metastasis.
EOC metastasis occurs from direct extension of the primary ovarian or fallopian tube tumors and exfoliation of single cells and multicellular aggregates (MCAs, or spheroids) into the peritoneal cavity, wherein dissemination is facilitated by the accumulation of ascites fluid (3). Metastatic MCAs are variable in size, number and integrity, and are highly prevalent in ascites. It has been demonstrated that patient-derived ascites MCAs adhere to live human mesothelial cell monolayers and sub-mesothelial extracellular matrix (ECM) in vitro (4,5). The free floating cells and MCAs that survive in peritoneal ascites fluid in vivo adhere to the mesothelial cells (MC) of the peritoneal membrane that covers abdominal organs, subsequently induce MC retraction, anchor in the collagen-rich sub-mesothelial extracellular matrix, and proliferate to form widely disseminated secondary lesions (3,6,7). Recently, an alternative hematogenous route for EOC metastasis to the ovary and peritoneal cavity with the development of ascites has been reported (8,9). However, the mechanisms that regulate widespread intra-peritoneal (i.p.) metastasis remain poorly understood.

The sub-mesothelial matrix is rich in interstitial collagen (3,6,7) and acts as a supportive scaffold to maintain tissue architecture as well as a physical barrier to metastatic implantation. Degradation of sub-mesothelial collagen, catalyzed by matrix metalloproteinases (MMPs), disrupts ECM structure and removes physical constraints on the cytoskeleton, enabling proliferation to anchor secondary lesions (10-12). MMPs are highly expressed in many tumors, and have been implicated in cellular migration, invasion, and metastasis (13,14). Membrane type 1 MMP (MT1-MMP, MMP-14) is a transmembrane proteinase that degrades interstitial collagen and other substrates and thereby plays a key regulatory role in modulating the peri-cellular microenvironment (10,15-19). In EOC, MT1-MMP can induce cell migration, cell-matrix detachment, ECM invasion, angiogenesis, MCA formation and shedding, and expansive growth within three-dimensional collagen matrices (20-23). MT1-MMP is not detected in normal ovarian epithelium or in benign ovarian tumors, but is highly expressed in later stage metastatic tumors (24), indicating that MT1-MMP may promote EOC metastasis through processing of peri-cellular substrates.

MT1-MMP is composed of a signal peptide, a prodomain that retains zymogen latency, a zinc-containing catalytic domain, two linkers surrounding a hemopexin domain, a transmembrane domain, and a short (20 amino acid) cytoplasmic tail. Increasing evidence suggests that the cytoplasmic tail of MT1-MMP regulates its activity at the cell surface. Endocytosis of MT1-MMP requires the cytoplasmic tail, and tail truncation restricts MT1-MMP to the cell surface (25-27). The cytoplasmic tail of MT1-MMP has three potential phosphorylation sites: Thr567, Tyr573, and Ser577. Tyr573 phosphorylation promotes retention of MT1-MMP on the cell surface and thereby enhances invasion of three-dimensional collagen gels (28,29). Alternatively, phosphorylation of Thr567 promotes detachment of cell-cell adherent sheets with subsequent expansive growth within 3D collagen gels (23). In the current study, we use MT1-MMP-T567E and -T567A phospho-mimetic and phospho-deficient mutants to evaluate the role of Thr567 phosphorylation in regulating the transition between free-floating ovarian cancer cells or MCAs and peritoneally anchored metastatic lesions. These data reveal a potential role for Thr567 phosphorylation in regulation of MCA dynamics, suggesting a contribution to overall metastatic success.

RESULTS

Expression of MT1-MMP Thr567 Mutants Alters E-Cadherin Integrity, Monolayer Cohesion, and Cell Motility — Biomimetic point mutations with naturally occurring amino acids are commonly used to study the effects of post-translational modifications such as phosphorylation. Phospho-mimetics add a negative charge to an amino acid that can mimic a phosphate group (Glu for Thr, for example) or alternatively prevent the ability to accept a phosphate group (Ala for Thr, for example) (30). Using this approach, phosphorylation of MT1-
MMP at Thr<sup>567</sup> of the cytoplasmic tail has been shown to promote expansive growth of invasive tumor cell colonies within constrained three-dimensional collagen gels (22,23,31,32). To investigate further the potential role of MT1-MMP-Thr<sup>567</sup> in cellular behaviors correlated to metastasis, GFP-tagged MT1-MMP phospho-mimetic (T567E, or TE) and phospho-defective (T567A, or TA) mutants as well as wild type MT1-MMP (MT) were expressed in OVCA433 cells, that have very low levels of endogenous MT1-MMP (Fig. 1A). Western blot data indicated equivalent expression levels of exogenous wild type or modified MT1-MMP (Fig. 1B, arrowhead). Note that, unlike endogenous phosphorylated MT1-MMP, the phospho-mimetic T567E mutant does not react with phospho-Thr antibodies (data not shown).

During cancer progression and metastasis, aggressive tumor cells lose epithelial features and gain a mesenchymal phenotype associated with enhanced motility and invasion. This epithelial-mesenchymal transition (EMT) process is often accompanied by decreased levels of E-cadherin, a critical epithelial cell-cell adhesion molecule (33-35). Transfection of MT1-MMP into OVCA433 cells results in a loss of intact E-cadherin and the appearance of a lower molecular weight degradation product (Fig. 1B, arrowhead). Cleavage was most pronounced in cells expressing the MT1-MMP-T567A mutant (Fig. 1B,C). Control blots were probed with β-actin to ensure equal protein loading.

To examine the potential functional consequences of E-cadherin cleavage in MT1-MMP-expressing cells, epithelial integrity was investigated using a dispase-based dissociation assay to assess the relative resistance to dissociation of epithelial cell sheets into single cells (Fig. 2A). Cells were cultured in the presence or absence of the broad spectrum MMP inhibitor GM6001, washed, treated with dispase and the detached sheets were subjected to controlled mechanical agitation followed by analysis of sheet fragmentation, with quantitation of both fragment number and fragment size (Fig. 2B, C; Table 1) (36). E-cadherin was examined by western blotting (Fig. 2D). Relative to MT1-MMP-expressing cells, parental OVCA433 cells retained better epithelial integrity, dissociating into fewer fragments. MT1-MMP expression decreased epithelial integrity (Fig. 2C) and enhanced cleavage of E-cadherin (Fig. 2D), leading to enhanced monolayer fragmentation. This was most pronounced in the phospho-deficient mutant T567A that dissociated into a large number of small fragments (Fig. 2C), indicative of reduced epithelial cohesion. This is consistent with western blot results (Fig.1B, C) showing lesser intact E-cadherin in MT1-MMP-T567A-expressing cells treated with GM6001.

Acquisition of the migratory phenotype is a key hallmark of tumorigenesis and is crucial to cancer metastasis (37,38). Using an in vitro cell migration assay to assess motility, cells expressing the phospho-mimetic MT1-MMP-T567E mutant were found to be highly migratory compared to cells expressing wild type MT1-MMP (Fig. 3A-C). This enhanced migratory capability was independent of cell proliferation, as our previous data demonstrated that MT1-MMP expression and Thr<sup>567</sup> modification enhances cell proliferation within a three dimensional collagen matrix, but not on a two-dimensional surface (22,23). In contrast, cells expressing the phospho-defective MT1-MMP-Thr<sup>567</sup>A mutant were not migratory (Fig. 3A-C). Lysis of wounded cultures and examination of E-cadherin and β1 integrin expression and processing by western blot did not reveal discernable differences in cell-cell or cell-matrix adhesion molecules (data not shown).

Expression of MT1-MMP Thr<sup>567</sup> Mutants Alters MCA Dynamics on Mesothelium and Collagen — The MCA is a key unit in metastatic dissemination of EOC; however molecular mechanisms that regulate MCA dynamics remain poorly understood. Non-adherent cells and MCAs in malignant ascites ultimately adhere to the mesothelium overlying peritoneal organs, anchor in sub-mesothelial collagen and proliferate to establish widely disseminated secondary lesions (3-6). We previously observed that MT1-MMP expression was higher in MCAs relative to 2-dimensional cultures (22). To investigate the
potential impact of MT1-MMP on MCA dynamics, EOC MCAs were produced using the hanging drop method from cells expressing wild type MT1-MMP or Thr<sup>567</sup> mutants and were evaluated by light and scanning electron microscopy (SEM) to assess aggregate area and overall morphology (Fig. 4A). In contrast to the mesenchymal cell line DOV13, that expresses high levels of endogenous MT1-MMP and grow as smooth spheroids, OVCA433 cells form poorly aggregated MCAs as visualized by light microscopy (Fig. 4B, first column). Similar to light microscopic observations, examination by high resolution SEM shows loosely clustered aggregates of cells in OVCA433 MCAs. Acquisition of MT1-MMP expression results in smaller, more cohesive spheroids. Cells expressing MT1-MMP-T567A formed the smallest spheroids. This may be due to less cell-cell aggregation, consistent with poor epithelial cohesion, as shown in Fig. 2. Additionally, we have recently shown that the presence of E-cadherin reduces spheroid cohesion in ovarian cancer MCAs (39).

Adhesion of exfoliated cells and MCAs to peritoneal mesothelial cells results in widespread diffuse peritoneal metastasis as tumor cells induce mesothelial cell retraction and expose the submesothelial collagen matrix to which ovarian cancer cells avidly anchor to form secondary lesions (3,6,7). To investigate whether MT1-MMP Thr<sup>567</sup> phosphorylation may affect MCA: mesothelial cell adhesion dynamics, we developed a novel three dimensional ex vivo adhesion assay (depicted in Fig. 4A) in which adhesion of fluorescent MCAs to the mesothelial surface of intact murine peritoneal explants is quantified (40-42). Using this approach, cells expressing the phospho-mimetic mutant MT1-MMP-T567E demonstrated a 4-fold increase in adhesion to peritoneal explants relative to cells expressing wild type or phospho-deficient MT1-MMP (Fig. 5A,B). Examination of adhesive MCAs by high resolution SEM imaging shows interaction of MCAs with the peritoneal mesothelial surface (Fig. 5C).

Underlying the monolayer of peritoneal mesothelial cells is a three-dimensional matrix rich in interstitial collagen (Fig. 6A,B). To examine kinetics of MCA dispersal on collagen, individual fluorescently tagged MCAs were seeded onto three-dimensional collagen and monitored for four days (Fig. 6C). Quantitative image analysis shows that MCAs comprised of cells expressing MT1-MMP-T567E dispersed more rapidly, while MCAs containing cells that expressed MT1-MMP-T567A did not disperse (Fig. 6D). Together with our previous data showing that MT1-MMP-T567E promotes expansive growth within mechanically constrained three-dimensional collagen gels (22), collectively these data support the hypothesis that phosphorylation of Thr<sup>567</sup> in the MT1-MMP cytoplasmic tail can promote ovarian cancer intra-peritoneal metastatic dissemination.

**DISCUSSION**

Ovarian cancer metastasis is initiated via the exfoliation of cells and MCAs into the peritoneal cavity. These anchorage-independent, chemotherapy-resistant cells and spheroids adhere to the monolayer of mesothelial cells covering peritoneal organs. Subsequently, mesothelial cell retraction is induced to facilitate cancer cell invasion into the interstitial collagen-rich submesothelial extracellular matrix, wherein they proliferate to anchor disseminated secondary lesions (3,6). In this unique niche, the molecular events that promote the transition from free-floating MCAs to intra-peritoneally anchored metastatic lesion are poorly understood.

The transmembrane collagenase MT1-MMP has been implicated in a multitude of pro-metastatic functions including tumor cell migration and invasion in constrained 3-dimension matrices (13,14,43,44). Increasing evidence shows that the cytoplasmic tail region of MT1-MMP functions as an important regulatory domain in modulating endocytosis and turnover, thereby controlling the expression of active MT1-MMP level at the cell surface (26,45,46). The cytoplasmic domain of MT1-MMP has three potential phosphorylation sites: Thr<sup>567</sup>, Tyr<sup>573</sup>, and Ser<sup>577</sup>. Src-dependent Tyr<sup>573</sup> phosphorylation of MT1-MMP plays a role in cell migration (28), while epidermal growth factor-induced
phosphorylation of Tyr573 regulates the transition between invasive and expansive growth in 3-dimensional collagen gels (29). Protein kinase C (PKC)-catalyzed phosphorylation of MT1-MMP at Thr567 regulates its intracellular trafficking and impacts cell migration and invasion in fibrosarcoma cells (32). In ovarian cancer cells, we recently demonstrated that a Thr567 phospho-mimetic mutant enhances matrix invasion and increases proliferation in 3D collagen gels (22,23).

In the current study, we further investigate the role of MT1-MMP cytoplasmic residue Thr567 phosphorylation in promoting EOC metastasis-associated behaviors. Our data show that Thr567 modulation alters monolayer cohesion, cell motility, and MCA dynamics. Smaller aggregates are seen with the T567A phospho-deficient mutant, indicating that MT1-MMP in the non-phosphorylated state may interact with cytoplasmic factors to promote spheroid compaction. Alternatively, this may be related increased to cleavage of E-cadherin in these cells, as E-cadherin reduced spheroid cohesion in ovarian cancer cells [39]. In contrast, cells expressing the phospho-mimetic T567E mutant adhere avidly to intact peritoneal explants and spread on the mesothelial cell surface, disrupting monolayer integrity and exposing the sub-mesothelial collagen matrix. Enhanced dissemination of T567E-expressing MCAs on collagen is also observed. Our group and others have reported less efficient internalization of the T567A mutant, leading to enhanced surface accumulation (versus increased internalization of the T567E mutant). Nevertheless, the T567E mutant is associated with enhanced migration, invasion and collagen degradation relative to the T567A mutant, suggesting that these functions are dependent on both MT1-MMP catalytic activity as well as internalization [23,27,32, current work].

These data are consistent with previous results in which pharmacologic inhibition or genetic silencing of integrin-linked kinase (ILK) was used to prevent phosphorylation of Thr567 in DOV13 cells, that endogenously express MT1-MMP (31). In this study, results observed were similar to those using cells transfected to exogenously express the phospho-defective mutant T567A in the current study. ILK is a serine/threonine kinase that is a cytoplasmic binding partner of β1- and β3-integrins, is activated by cell-matrix adhesion, and is highly expressed in ovarian cancer (47-49). We recently reported that ILK is co-expressed with MT1-MMP in EOC cells and tissues and can catalyze phosphorylation of Thr567 (31). Furthermore, silencing of ILK expression with siRNA or reduction of ILK activity with the small molecule inhibitor QLT0267 significantly inhibits adhesion to and invasion of both meso-mimetic cultures and collagen gels by EOC cells (31). Taken together with the results of current study, these findings suggest that post-translational regulation of the MT1-MMP cytoplasmic tail may function as a fine tuned regulatory mechanism to impact ovarian cancer metastatic success and indicate that further evaluation of this process is warranted in preclinical studies of ovarian cancer metastasis.

EXPERIMENTAL PROCEDURES

Materials — The immunological reagents used were anti-MT1-MMP antibodies (#ab51074 [EP1264Y], Abcam, Cambridge, MA), anti-E-Cadherin (#ab40772 [EP700Y], Abcam), and anti-β-Actin–peroxidase (#A3854, clone AC-15, Sigma-Aldrich, St. Louis, MO). Rat tail collagen type I was purchased from BD Biosciences (San Diego, CA). Immobilon-P polyvinylidene membranes were purchased from Millipore (Billerica, MA). SuperSignal West Dura Chemiluminescent Substrate was purchased from Thermo Scientific (Rockford, IL). SYLGARD® 184 Silicone Elastomer Kit was purchased from Dow Corning (Midland, MI). Geneticin® (G418) was obtained from Life Technologies (Carlsbad, CA). The broad spectrum MMP inhibitor GM6001 was purchased from EMD Millipore (Billerica, MA).

Cell Culture — The ovarian cancer cell lines OVCA433 and DOV13 were provided by Dr. Robert Bast (M.D. Anderson Cancer Center, Houston, TX) and maintained in Minimal Essential Medium (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum
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(Gibco), 1% non-essential amino acids (Corning), 1 mM sodium pyruvate (Corning), 0.1% amphotericin B (Corning), 100 U/ml penicillin and 100 \( \mu \)g/ml streptomycin (Lanza). DOV13 medium was cultured in the above media supplemented with 10\( \mu \)g/ml Insulin (Gibco). The transfection and generation of the stable cell lines OVCA433-MT-GFP, OVCA433-T567E-GFP, and OVCA433-T567A-GFP (GFP-tagged in the linker region prior to the transmembrane domain) have been previously described (22,23). Transfected cells were maintained in media supplemented with 600 \( \mu \)g/ml Geneticin.

**Western Blotting** — Cells were lysed and collected using mRIPA lysis buffer (1% Triton X-100, 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 20 mM NaF, 10 mM Na\textsubscript{2}P\textsubscript{2}O\textsubscript{7}) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN), and protein concentration was determined using the Bio-Rad DC protein assay kit (Hercules, CA). Cell lysates (20 \( \mu \)g) were electrophoresed on 9% SDS-PAGE gel electrophoresis, transferred to Immobilon-P PVDF Membrane (EMD Millipore) using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad), and blocked with 3% bovine serum albumin (BSA) in TBST buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature. Membranes were incubated overnight at 4\(^\circ\)C with anti-MT1-MMP antibodies (#ab51074 [EP1264Y], 1:2,000, Abcam), or anti-E-Cadherin (#ab40772 [EP700Y], 1:5,000, Abcam). The immunoreactive proteins were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:4,000, Sigma-Aldrich, St. Louis, MO) followed by enhanced chemiluminescent developing with SuperSignal West Dura extended duration substrate (Thermo Fisher Scientific, Kalamazoo, MI) using an ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences, Pittsburgh, PA). Protein blots were then stripped and rebotted with anti-\( \beta \)-Actin–peroxidase antibody (#A3854, clone AC-15, 1:100,000, Sigma-Aldrich) to ensure similar amounts of protein were present in each lane. Protein band intensities were quantified by densitometric analysis using ImageJ software (gel analysis tool) available at https://imagej.nih.gov/ij/, and values were normalized to \( \beta \)-actin loading control and expressed as a ratio. The assay was repeated in three independent biological replicates and statistically significant differences were determined using one-way analysis of variance (ANOVA).

**Analysis of Epithelial Cohesion** — Cell-cell cohesion was evaluated using a dispase-based dissociation assay as previously described (36). Specifically, cell lines (OVCA433, OVCA433-MT-GFP, OVCA433-T567E-GFP, and OVCA433-T567A-GFP) were cultured in 60-mm dishes to 90% confluence in the presence or absence of the broad-spectrum MMP inhibitor GM6001 (25 \( \mu \)M or 50 \( \mu \)M), washed twice with PBS to remove GM6001, and incubated in 2 ml of dispase solution (STEMCELL Technologies, Cambridge, MA) until the cell monolayer was detached. Released cell sheets were washed twice with PBS, transferred to 15 ml conical tubes in a total volume of 3 ml PBS, and subsequently subjected to 50 inversion cycles on a rotator. The dissociated cellular fragments were transferred to a tissue culture plate and imaged with Leica M60 stereo microscope (Buffalo Grove, IL). Cellular fragments were analyzed using ImageJ and categorized as small (0.02-0.1\( \text{mm}^2 \)), medium (0.11-0.5\( \text{mm}^2 \)) or large (>0.51\( \text{mm}^2 \)) according to the fragment size. The total number of fragments in each category were manually counted using ImageJ cell counter plugin tool for all cell lines. Results shown represent three independent biological replicates and statistically significant differences were determined using one-way ANOVA.

**Analysis of Cell Motility** — An Ibidi culture insert (Ibidi GmbH, Martinsried, Germany) consists of two reservoirs separated by a 500 \( \mu \)m thick wall. To evaluate cell motility in vitro, an Ibidi culture insert was placed into one 35 mm dish prior to seeding both reservoirs with EOC cells (30,000 cells in 70 \( \mu \)l volume) and incubation at 37\(^\circ\)C in 5% CO\textsubscript{2}. After 16 hours, the insert was gently removed creating a gap of \( \sim \)500 \( \mu \)m, which allows the cells in each compartment to migrate towards each other. Following removal of the insert, cells were gently washed with PBS and incubated in serum free media. Cells were
photographed using an EVOS® FL Cell Imaging System (Thermo Fisher Scientific) at the time of insert removal (0 hr), and cell migration was observed following 5 hr and 24 hr of incubation. The cell migration rate and percentage of gap closure area were quantified by analyzing images using ImageJ. Results shown represent three independent biological replicates and statistically significant differences were determined using one-way ANOVA. In some experiments, wounded cultures were lysed and examined by western blotting for changes in E-cadherin and MT1-MMP expression and processing.

**Multiple Cellular Aggregate (MCA) Formation** — MCAs were generated using a hanging drop method as previously described (22). Specifically, EOC cells (300 - 2,000 in 20 μl droplets) were individually seeded on a culture dish lid prior to gentle inversion of the lid and culture as hanging drops at 37°C for 2 days to allow MCAs formation. MCAs were imaged with Olympus BX43 light microscopy, and with FEI-MAGELLAN 400 FESEM scanning electron microscopy as described below. MCA formation assay were done three times in ten replicates each time for every cell line. MCA areas (µm²) were measured using ImageJ and statistical analysis was conducted using one-way ANOVA.

**MCA Dispersal on Collagen** — Tissue culture plates were coated overnight at 4°C with type I rat tail collagen (BD Biosciences, San Jose, CA) at 5 μg/cm² in sodium carbonate (pH 9.6). MCAs (300 cells in 20 μl droplets) were individually transferred into one well of collagen coated plate. Dynamic dispersal patterns were imaged at various time points from 1-4 days using EVOS® FL Cell Imaging System (Thermo Fisher Scientific). Results shown represent three independent biological replicates. MCA area (µm²) was measured using ImageJ and the MCA area ratio was normalized by dividing each day’s MCA area by the Day1 MCA area for each cell line. Statistical analysis was performed using one-way ANOVA.

**Ex vivo Peritoneal Explant Adhesion Assay** — Immediately prior to MCA formation, cell lines (OVCA433, OVCA433-MT-GFP, OVCA433-T567E-GFP, and OVCA433-T567A-GFP) to be assayed were stained with 2 μM CellTracker Green CMFDA (Molecular Probes, Eugene, OR) for 30 min. MCAs (300 cells per 20ul droplets) were generated via the hanging drop method. C57BL/6 female mouse peritonea were excised, rinsed in PBS twice and trimmed into two pieces of approximately 1.5×1.5 cm². Approximately 300 individual MCAs from each cell line were added to each explant with the mesothelial surface facing up. To minimize the individual mouse peritonea difference, for each mouse the left side peritoneum was always loaded with Ovca433 MCAs as an internal normalization control, and the right side peritoneum was used for loading MCAs from each mutant cell line. MCA-loaded-peritonea were incubated for 4 h at 37°C, and then vigorously washed in PBS (five times) followed by mounting onto a glass coverslip (with the mesothelial surface facing down). Adherent MCAs were imaged with EVOS FL Cell Imaging System for the whole peritoneum. MCA area (µm²) was determined using ImageJ to measure all images, generating a total area occupied for each cell line. This was then normalized with the Ovca433 MCA total area of the same mouse using the left side peritoneum. The assay was repeated in three biological replicates for all cell lines, and statistical analysis was performed using one way ANOVA. Additional explants were processed for scanning electron microscopy (50) as described below.

**Scanning Electron Microscopy (SEM)** — MCAs were generated using the hanging drop method as described above and collected by centrifugation at 4,000 rpm for 15 min. The resulting MCAs were re-suspended in primary fixative solution (2% glutaraldehyde, 2% paraformaldehyde in 0.1M cacodylate buffer pH 7.35) and rotated for 1 hr at room temperature. After washing with 0.1M cacodylate buffer for 5 min, MCAs were pelleted by centrifugation, re-suspended in 100 μl 0.1M cacodylate buffer, and loaded onto poly-L-lysine coated cover slips for 30 min at room temperature. Secondary fixation was performed with 2% osmium tetroxide in 0.1 M cacodylate buffer in a PELCO EM Pro vacuum
chamber inside a PELCO BioWave Pro microwave tissue processor (Ted Pella, Redding, CA). Samples were then rinsed with 0.1 M cacodylate buffer, washed with ultrapure water 3 x 5 min, and dehydrated through a series of increasing concentration of ethanol (20%, 50%, 70%, 90%, 3 x 100%). Critical point drying was processed with Tousimis AutoSAMDR-931 critical point dryer (Micro to Nano, Haarlem, Netherlands). Completely dried samples were placed on carbon stubs and coated with FlashDryTM silver paint. After sputter coating with iridium, samples were examined under FEI-Magellan 400 field emission scanning electron microscope (FEI, Hillsboro, OR) in the Notre Dame Integrated Imaging Facility. For SEM processing of mice peritoneal explants with or without MCAs, peritoneal tissue was fixed in primary fixative solution overnight at 4°C, followed by 3 x 20 min washes in 0.1 M Cacodylate buffer before secondary fixation and further processing as described above. A minimum of 10 MCAs per cell type were imaged.

**Statistical Analysis** — Experiments were repeated for at least three times independently. The statistical analysis of the data was done using OriginPro 9.0 (Northampton, MA) software. Comparison between groups was performed using one-way analysis of variance (ANOVA) to determine p values. p < 0.05 was accepted as level of significance. * significant p < 0.05; ** highly significant p < 0.01.

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**Author Contributions:** JY coordinated the study, performed and/or supervised the experiments, and wrote the manuscript. WCK performed experiments and analyzed data for Figs. 1, 3, 5, and 6. AC performed the experiments and analyzed data for Fig. 2. ZL assisted with the experiments in Figs. 5-6. KQ assisted with the experiments in Fig. 2. MSS conceived of the study, analyzed all data, and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.
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FIGURE LEGENDS

FIGURE 1. Expression of MT1-MMP cytoplasmic tail mutants in ovarian cancer cells. (A) Schematic illustration of wild-type and cytoplasmic tail mutations of MT1-MMP. Structural domains of MT1-MMP are shown from the N terminus: (SP) signal peptide; (Pro) pro-peptide that contains a basic RRKR motif cleaved by furin; (CAT) catalytic domain that contains the zinc-binding site (Zn$^{2+}$); (H) hinge region; (HPX) Hemopexin-like domain; (L) linker; (TM) Transmembrane domain; and (CT) cytoplasmic tail domain (20-amino acids). Thr$^{567}$ (T) in the cytoplasmic tail was mutated either to Glu (E) to make a phospho-mimetic mutant (T567E, or TE), or to Ala (A) to generate a phospho-defective mutant (T567A, or TA) [22,23]. (B) Cell lines were generated in an OVCA433 parental background expressing GFP-tagged wild type MT1-MMP (MT), MT1-MMP-T567E (TE), and MT1-MMP-T567A (TA). Western blotting was performed to detect MT1-MMP, E-cadherin (E-cad), and β-actin as a loading control. Cell lysates (20 µg) were electrophoresed on 9% SDS-polyacrylamide gels and electroblotted to Immobilon as described in Experimental Procedures. Blots were probed with anti-MT1-MMP (1:2000 dilution), or anti-E-cadherin (1:5000 dilution) as indicated and developed using a peroxidase conjugated secondary antibody (1:4000 dilution) and ECL detection, as described in Experimental Procedures. Blots were stripped and re-probed with or anti-β-actin-peroxidase antibody (1:100,000 dilution) to ensure equal loading. (upper panel) arrow, 55 kDa active MT1-MMP; arrowhead: 82 kDa GFP tagged MT1-MMP. (second panel) arrow, 120 kDa intact E-cadherin; arrowhead: 100 kDa cleaved E-cadherin. (C) Densitometric analysis of cleaved E-cadherin. Experiments were repeated in biologic triplicates. ** p<0.01.

FIGURE 2. Effect of MT1-MMP expression on epithelial cohesion. (A) Confluent cell-cell adherent monolayers were separated from culture dishes as cell-cell adherent sheets by pulsing with dispase until sheets detached from the dish. (B) Cells were cultured to confluency in the absence or presence of 50 µM GM6001 (broad-spectrum MMP inhibitor), washed extensively, and incubated in 2 ml of dispase solution until the cell monolayer was detached. Released cell sheets were subjected to mechanical disruption using 50 inversion cycles on a rotator. The dissociated cellular fragments were imaged with Leica M60 stereo microscope. (C) Cellular fragments were analyzed using ImageJ and categorized as small (0.02-0.1mm²),
medium (0.11-0.5 mm²) or large (>0.51 mm²) according to the fragment size. The total number of fragments in each category was manually counted. Results shown represent three independent biological replicates. (D) Cells were cultured to confluence in the absence “-”, or presence of GM6001 (25 µM “+”, or 50 µM “++”), washed, and incubated in dispase until the cell sheet was released, followed by rotation for mechanical disruption. The dissociated cellular fragments were lysed in mRIPA buffer, and Western blotting was performed to detect E-cadherin (E-cad). β-actin was used as a loading control.

FIGURE 3. Phospho-mimetic MT1-MMP-T567E enhances cell migration. (A) Cells were grown using a cell culture insert containing two reservoirs blocked by the insert wall. The insert was removed to allow cell migration between the reservoirs, and cells were photographed at various time points up to 24 hours. (B) Rate indicates the average distance traveled by the cell front at each time point relative to the 0 hour time point. p<0.01 for the comparisons TE vs MT, TE vs TA and MT vs TA. (C) Percentage closure indicates the area fraction of the space at the 0 hour time point that has been covered by migrating cells. p<0.01 for the comparisons TE vs MT, TE vs TA and MT vs TA. Experiments were repeated three times independently.

FIGURE 4. Expression of MT1-MMP mutants alters MCA morphology. (A) Diagram depicting MCA generation via the hanging drop method. Cells (300 – 2000 in 20 ul) were seeded onto the top of 35 mm culture dishes, then gently inverted and allowed to aggregate for 2 days. MCAs were subsequently imaged or used in an ex vivo peritoneal explant adhesion assay (described in Fig. 5). (B) MCAs were imaged using Olympus BX43 light microscopy (first column) or with FEI-MAGELLAN 400 FESEM scanning electron microscopy as described in Experimental Procedures. Magnification is as indicated. (C) MCA area (µm²) was measured using ImageJ from images acquired using Olympus BX43 light microscopy. Scatter plot represent the area measurement of n=10 MCAs. p < 0.05 was accepted as level of significance. ** p < 0.01.

FIGURE 5. Phospho-mimetic MT1-MMP-T567E mutant enhances adhesion of MCAs to intact ex vivo peritoneal explants. (A) Peritoneal tissue was dissected from female mice (as depicted in Fig. 4A) and pinned mesothelium-side up on optically clear silastic resin. MCAs were generated from fluorescently tagged cells via the hanging drop method and were distributed evenly on excised peritonea (~300 MCAs per explant). After 4h, adhesion of MCAs to peritoneal tissue was quantified by fluorescence microscopy using the EVOS FL Cell Imaging System. (B) Adherent MCAs were imaged with EVOS FL Cell Imaging System for the whole peritoneum. To minimize the individual mouse peritonea difference, for each mouse the left side peritoneum was always loaded with Ovca433 MCAs as an internal normalization control, and the right side peritoneum was used for loading MCAs from each mutant cell line. MCA area (µm²) was determined using ImageJ, summed as a total area for each cell line, and normalized with Ovca433 MCAs total area that using the same mouse left side peritoneum. The assay was repeated in three biological replicates, and statistical analysis was performed using one-way ANOVA. ** p < 0.01. (C) MCA interaction with peritoneal explants was imaged using FEI-MAGELLAN 400 FESEM scanning electron microscopy as described in Experimental Procedures.
FIGURE 6. Phospho-mimetic MT1-MMP-T567E mutant exhibits enhanced dispersal on intact ex vivo peritoneal explants and collagen surfaces. (A,B) Peritoneal tissue was dissected from female mice as depicted in Fig. 4A and pinned mesothelium-side up on optically clear silastic resin. MCAs were generated via the hanging drop method from parental Ovca433 cells or Ovca433 cells expressing wild type MT1-MMP (MT), MT1-MMP-T567E (TE), or MT1-MMP-T567A (TA) and were distributed evenly on excised peritonea. Peritoneal explants were imaged using FEI-MAGELLAN 400 FESEM scanning electron microscopy as described in Experimental Procedures. Image in (A) shows only sub-mesothelial collagen matrix while (B) shows the boundary (yellow line) of an MCA interacting with collagen. (C) MCAs were transferred independently into single wells of a plate containing collagen gels and dispersal patterns were imaged daily for four days using EVOS® FL Cell Imaging System. A representative time series of dispersal of an MCA comprised of MT1-MMP-T567E-expressing cells is shown. (D) Quantitative image analysis was performed using ImageJ to quantify MCA dispersal at each time point. MCA area ($\mu m^2$) was measured using ImageJ, and MCA area ratio was normalized by dividing each day MCA area over Day1 MCA area for each cell line. Results shown represent three independent biological replicates. Statistical analysis was performed using one-way ANOVA. Day 2 comparisons, $p<.05$ for TE vs Ovca433, TE vs MT, and TE vs TA. Day 4 comparisons, $p<.05$ for TE vs Ovca433 and TE vs TA.
**Table 1: Dispase Assay Fragment Analysis.** Confluent monolayers were pulsed with dispase to release cell-cell adherent sheets and incubated in the presence or absence of the broad spectrum MMP inhibitor GM6001 (50µM) or DMSO control, as indicated, for 50 inversion cycles on a rotator. Dissociated fragments were imaged and enumerated as described in Experimental Procedures. Fragment sizes were arbitrarily categorized as Large: > 0.51mm²; Medium: 0.11-0.5mm²; Small: 0.02-0.1 mm².

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ovca433</th>
<th>MT</th>
<th>TE</th>
<th>TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM6001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large</td>
<td>24 ± 2</td>
<td>22 ± 2</td>
<td>13 ± 2</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Medium</td>
<td>65 ± 4</td>
<td>29 ± 6</td>
<td>77 ± 7</td>
<td>65 ± 2</td>
</tr>
<tr>
<td>Small</td>
<td>96 ± 3</td>
<td>40 ± 9</td>
<td>162 ± 6</td>
<td>68 ± 4</td>
</tr>
</tbody>
</table>

a1 statistically compared to the Ovca433/DMSO control/Large fragment group; b1 statistically compared to the TE/DMSO control/Large fragment group; c1 statistically compared to the TE/DMSO control/Medium fragment group; d1 statistically compared to the Ovca433/DMSO control/Medium fragment group; e1 statistically compared to the Ovca433/DMSO control/Small fragment group; f1 statistically compared to the TA/DMSO control/Small fragment group; a2 statistically compared to the Ovca433/GM6001/Large fragment group; b2 statistically compared to the MT/GM6001/Large fragment group; c2 statistically compared to the Ovca433/GM6001/Medium fragment group; d2 statistically compared to the Ovca433/GM6001/Small fragment group; e2 statistically compared to the TA/GM6001/Small fragment group; a3 statistically compared to the MT/DMSO control/Large fragment group; b3 statistically compared to the MT/DMSO control/Small fragment group; c3 statistically compared to the TE/DMSO control/Small fragment group; d3 statistically compared to the TA/DMSO control/Large fragment group; e3 statistically compared to the TA/DMSO control/Medium fragment group; * statistically significant difference (p<0.05).

** Statistically significant difference defined as p<0.01.
Yang et al., Fig. 1

A

B

C

Dov13
Ovca433
MT
TE
TA

MT1-MMP

E-Cadherin

β-actin

MT1-MMP

Cleaved E-Cadherin

Ovarian Cancer Cell Lines

Relative Intensity

0 1 2 3 4 5 6

0 1 2 3 4 5 6

RRHGTPRRLLYCQRSSLKDVK

TE: Thr567(T) to Glu(E) Phospho-mimetic mutant
TA: Thr567(T) to Ala(A) Phospho-defective mutant

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Yang et al., Fig. 3

Migration at 24 h

Wound closure at 24 h

Rate (µm/hr)

Closure (%)

0 hr

5 hr

24 hr

MT

TE

TA

0%

20%

40%

60%

80%

100%
Yang et al., Fig. 4

A

B

Light Microscopy

Dov13

Ovarian Cancer Cell Lines

MCAs Size

Dov13  Ovca433  MT  TE  TA

Scanning Electron Microscopy

2,500 x  10,000 x  30,000 x

**  **  **

Ovary Cancer Cell Lines

MCAs Area [µm²]

0  50,000.0  100,000.0  150,000.0  200,000.0  250,000.0

0  1  2  3  4  5  6
A

B

C

Yang et al., Fig. 5

MT

TE

TA

400 µm

100 µm

400 µm

400 µm

100 µm

100 µm

100 µm

50 µm

50 µm

50 µm

50 µm

30 µm

30 µm

30 µm

30 µm

10 µm

10 µm

10 µm

10 µm

5 µm

5 µm

5 µm

5 µm

Aovca433

MT

TE

TA

Day 1

Day 4

Day 3

Day 2

MCAs on Mouse Peritoneum (%)

Ovarian Cancer MCAs

**

**

MT

TE

TA

Ovca433

400 µm

400 µm

400 µm

400 µm

100 µm

100 µm

100 µm

100 µm

50 µm

50 µm

50 µm

50 µm

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