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Membrane-type I Matrix Metalloproteinase-Dependent Ectodomain Shedding of Mucin16/CA-125 on Ovarian Cancer Cells Modulates Adhesion and Invasion of Peritoneal Mesothelium

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Abstract

Mucin16 (MUC16/cancer antigen 125 (CA-125)), a high molecular weight glycoprotein expressed on the ovarian tumor cell surface, potentiates metastasis via selective binding to mesothelin on peritoneal mesothelial cells. Shed MUC16/CA-125 is detectable in sera from ovarian cancer patients. We investigated the potential role of membrane-type 1 matrix metalloproteinase (MT1-MMP, MMP-14), a transmembrane collagenase highly expressed in ovarian cancer cells, in MUC16/CA-125 ectodomain shedding. An inverse correlation between MT1-MMP and MUC16 immunoreactivity was observed in human ovarian tumors and cells. Further, when MUC16-expressing OVCA433 cells were engineered to overexpress MT1-MMP, surface expression of MUC16/CA-125 was lost, while cells expressing the inactive E240A mutant retained surface MUC16/CA-125. As a functional consequence, decreased adhesion of cells expressing catalytically active MT1-MMP to three-dimensional mesomimetic cultures and intact *ex vivo* peritoneal tissue explants was observed. Nevertheless, mesomimetic invasion is enhanced in MT1-MMP-expressing cells. Together these data support a model wherein acquisition of catalytically active MT1-MMP expression in ovarian cancer cells induces MUC16/CA-125 ectodomain shedding, reducing adhesion to meso-mimetic cultures and to intact peritoneal explants. However, proteolytic clearing of MUC16/CA-125, catalyzed by MT1-MMP, may then expose integrins for high affinity cell binding to peritoneal tissues, thereby anchoring metastatic lesions for subsequent proliferation within the collagen-rich sub-mesothelial matrix.

Keywords

biomarker; meso-mimetic; metastasis; MMP-14; proteolysis

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INTRODUCTION

The ovarian surface epithelium (OSE) is the modified pelvic mesothelium covering the ovary and is comprised of a single layer of non-distinguished flat-to-cuboidal epithelial cells that are separated by a single basement membrane from the underlying ovarian stroma (Auersperg et al. 2001; Cannistra 2004). Nearly 90% of human ovarian cancers are epithelial in origin and epithelial ovarian cancer (EOC) is the sixth leading cause of overall cancer death for women in developed countries; however, the etiology and early events in EOC progression to metastasis remain poorly understood (Jemal et al. 2011).

Despite their epithelial morphology, OSE cells also display mesenchymal characteristics, including the production of mesenchymal metalloproteases and cadherins and the lack of epithelial cadherins (Auersperg et al. 1999; Strauss et al. 2011). This phenotypic plasticity further complicates identification of the specific molecular events occurring during EOC metastatic progression, which has historically been presumed to arise as a consequence of malignant transformation and proliferation of epithelial cells from the OSE (Fathalla 1971). Several recent studies have provided an alternative hypothesis, suggesting that the initiating epithelial cells involved in formation of high-grade serous ovarian cancer are derived from non-ovarian sources, such as the endocervix and fallopian tubes (Dubeau 2008; Berns and Bowtell 2012; Hillier 2012; Kim et al. 2012). Regardless of the site of origin, EOC metastasis involves detachment of epithelial cells from the primary tumor and dissemination into the peritoneal cavity as single cells and multicellular aggregates (MCA), which then adhere to and anchor in the mesothelial cell monolayer that lines the peritoneal cavity, to subsequently proliferate within the interstitial collagen-rich sub-mesothelial matrix and establish secondary lesions (Hudson et al. 2008; Barbolina et al. 2009). Elucidating the early molecular mechanisms involved in this metastatic process, specifically the adhesion of EOC cells to mesothelial cells and penetration of the associated submesothelial extracellular matrix, is essential to the development of future therapeutic agents.

Mucin16 (MUC16), also known as cancer antigen 125 (CA-125), is a large cell surface bound mucinous glycoprotein (molecular weight >2.5 million dalton). This heavily glycosylated mucin, containing both *O*-linked and *N*-linked oligosaccharides, is not detected in the epithelium of normal ovaries but is highly expressed on the ovarian tumor cell surface, suggesting a physiologic role in EOC (Yin et al. 2002; Kui Wong et al. 2003). Reported biological functions include cytolytic inhibition of human natural killer cells, facilitation of tumor cell-to-mesothelial cell adhesive interaction on peritoneal surfaces, and modulation of tumor cell growth (Rump et al. 2004; Patankar et al. 2005; Gubbels et al. 2006; Thériault et al. 2011). MUC16 has been shown to bind strongly to mesothelin, a protein present on peritoneal mesothelial cells (Rump et al. 2004; Gubbels et al. 2006; Scholler et al. 2007) and this interaction is thought to facilitate the initial adhesion and subsequent implantation and peritoneal spread that characterize EOC metastasis. An uncharacterized proteolytic event catalyzes shedding of MUC16 from the tumor cell surface, whereupon the shed ectodomain can be measured in peritoneal fluid and circulating blood (Vergote et al. 1992; Goodell et al. 2009). This shed form is detected in the serum of EOC patients as CA-125 and is considered to be the standard molecular marker of EOC malignancy (Bast et al. 1983; Scholler and Urban 2007; Moore et al. 2010).

Membrane type 1 matrix metalloproteinase (MT1-MMP, MMP-14) is a transmembrane proteinase that degrades interstitial collagen as well as a number of other substrates (Barbolina et al. 2007). Expression and activity of MT1-MMP have been implicated in a number of pro-metastatic events including proliferation, adhesion, invasion and metastasis (Egeblad and Werb 2002; Sounni et al. 2002; Doi et al. 2011). MT1-MMP is not detected in normal ovarian surface epithelium nor in benign ovarian tumors, but is widely expressed in ovarian carcinomas of all histologic types (Sakata et al. 2000). In EOC, MT1-MMP expression has been shown to promote cell migration, extracellular matrix invasion, angiogenesis, MCA formation, and expansive growth within 3D collagen matrices (Barbolina et al. 2007; Moss et al. 2009b; Kaimal et al. 2013). We have previously presented evidence to suggest that MT1-MMP activity at the cell surface can induce cell-matrix detachment, as well as promote the formation and shedding of metastatic MCAs in EOC (Moss et al. 2009a). The overexpression of MT1-MMP in EOC tumors relative to the normal ovary, with enhanced expression in metastases relative to primary tumors (Barbolina et al., 2007), suggests that it may catalyze cell surface proteolytic events that contribute to EOC metastatic success.

In the current study, we evaluated the relationship between MT1-MMP and MUC16 in heterotypic adhesive and invasive events to mimic initial events in EOC metastasis. Results suggest that MT1-MMP activity correlates with decreased cell surface expression of MUC16 and altered adhesion to mesothelial cells and tissues.

RESULTS

Inverse relationship between MT1-MMP and MUC16 expression in ovarian cells and tissues

MT1-MMP expression is elevated in ovarian tumors and increases in metastatic lesions (Barbolina et al. 2007; Moss et al. 2009b; Kaimal et al. 2013). To assess the potential correlation between MUC16 and MT1-MMP, expression of both antigens was assessed in human ovarian tumors as well as in ovarian cancer cells. Serial sections of a tumor tissue microarray consisting of 50 cores of ovarian adenocarcinoma were subjected to immunohistochemical analysis for either MT1-MMP (Fig. 1A–C) or MUC16 (Fig. 1D–F) and representative examples are shown. A trend toward an inverse relationship between MT1-MMP and MUC16 expression is observed, wherein tissues with higher level MT1-MMP expression (Fig. 1C) exhibit low MUC16 (Fig. 1F) while tissues with elevated MUC16 (Fig. 1D) do not stain positively for MT1-MMP (Fig. 1A). Of the cores exhibiting moderate to high MUC16 staining, 60% scored negative to low for MT1-MMP. Similar results were observed in cultured ovarian cancer cells (Fig. 2). Using OVCAR3, an ovarian cancer cell line known to express high levels of MUC16 (Yin and Lloyd 2001), no surface expression of MT1-MMP was observed (Fig. 2A–B). Similar results were obtained with parental OVCA433 cells (Fig. 2C–D). However when OVCA433 cells were engineered to overexpress catalytically active MT1-MMP (designated OVCA433-MT), surface staining for MUC16 was lost (Fig. 2E–F). Expression of a catalytically inactive mutant of MT1-MMP (E240A substitution), which represents a functional loss of MT1-MMP activity, restored MUC16 immunoreactivity (Fig. 2G–H). Real-time PCR analysis demonstrated equivalent expression of MUC16 mRNA across OVCA samples (Fig. 3A). However evaluation of

surface MUC16 levels using flow cytometry showed diminished surface MUC16 in OVCA433-MT cells relative to parental OVCA433 (Fig. 3B), suggesting post-translational modification. This was supported by data showing that MUC16 surface expression was restored in cells cultured with the broad spectrum MMP inhibitor GM6001 or in cells transfected with the inactive MT1-MMP active site mutant OVCA433-MT-E240A. We also stained for MUC1, another ovarian tumor cell surface bound mucin that is released into the circulation and is employed as a biomarker (Devine et al. 1992). No significant change in expression was found in OVCA433-MT cells compared to control (data not shown).

As the data above demonstrate an inverse relationship between MT1-MMP and MUC16, suggesting that MT1-MMP may participate in MUC16 ectodomain shedding, conditioned medium from MT1-MMP-expressing cells were analyzed for the presence of shed MUC16. Parental OVCA433 cells, OVCA433-MT, and OVCA433-E240A cells were each cultured for 48 hours, after which the conditioned media were assessed for human CA-125 using a commercially available ELISA. While quantifiable CA-125 was found in all samples (Fig. 3C); the concentration of CA-125 found in conditioned medium from OVCA433-MT cells was significantly increased compared to controls. Shed CA-125 levels were decreased in cells cultured with GM6001 and were below control levels in OVCA433-MT-E240A cells.

Expression of MT1-MMP alters ovarian cancer cell:mesothelial cell adhesion and invasion

Peritoneal mesothelial cells express high levels of mesothelin and MUC16 has been shown to interact strongly with mesothelin, with binding occurring via the N-linked glycoproteins of cell surface bound MUC16 (Gubbels et al. 2006). To evaluate whether MT1-MMP-catalyzed MUC16 ectodomain shedding alters the ability to bind mesothelin, a heterotypic cell adhesion assay was used. In this assay, fluorescently labeled ovarian cancer cells were incubated with confluent monolayers of adherent human peritoneal mesothelial cells (LP9) plated atop a collagen type I matrix, washed and enumerated (Fig. 4A). While parental OVCA433 cells adhered avidly to mesothelial cells (Fig. 4B), mesothelial adhesion was significantly reduced in OVCA433-MT cells. Mesothelial adhesion was significantly restored in cells expressing catalytically inactive MT1-MMP-E240A or by treatment of cells with the broad spectrum MMP inhibitor GM6001.

We have previously shown that expression of MT1-MMP enhances the ability of ovarian cancer cells to invade collagen type I gels (Ellerbroek et al. 2001; Moss et al. 2009b). To evaluate the role of MT1-MMP in penetration of a more physiologically relevant cellmatrix context, the ability to invade three-dimensional meso-mimetic cultures cultured atop porous membranes in a transwell was evaluated (Fig. 4C; Lengyel et al. 2013). Expression of MT1-MMP significantly enhanced meso-mimetic invasion (Fig. 4D), while cells expressing the catalytically inactive MT1-MMP-E240A were less invasive than control OVCA433 cells.

Tumor cell interaction with intact peritoneal mesothelium was also assessed using an *ex-vivo* assay to examine fluorescent ovarian cancer cell attachment to live mesothelial tissue. In this experiment, cells are incubated with immobilized peritoneal explants and adhesion is quantified by scanning electron microscopy or by fluorescence microscopy (Fig. 5A–F). Relative to control OVCA433 cells, attachment of OVCA433-MT cells to the peritoneal

explant was decreased. Adhesion to the peritoneal explant was significantly restored in cells expressing the catalytically inactive MT1-MMP-E240A mutant (Fig. 5G).

DISCUSSION

The primary cellular target for ovarian cancer metastasis is the mesothelial cell, which covers the peritoneum lining the peritoneal cavity. Ovarian cancer cells dissociated from the primary tumor metastasize intra-peritoneally through adhesion to and localized invasion of peritoneal mesothelium to anchor secondary lesions. Over 70% of EOC are diagnosed with intra-peritoneal metastasis, when 5-year survival rates are less than 30%. However, according to the most recent *SEER* database report, when EOC are diagnosed prior to metastatic dissemination, the survival rate dramatically increases to 92% (Howlader et al. 2013). Numerous studies investigating serum biomarkers to screen women at risk for EOC have assessed several potential markers but none are considered to have sufficient sensitivity and specificity for effective population-level early detection (Cramer et al. 2011; Husseinzadeh 2011; Mai et al. 2011).

MUC16/CA-125, a cell surface glycoprotein, is highly expressed on ovarian tumors whereupon it is shed from the tumor surface via a proteinase-dependent mechanism. MUC16/CA-125 in the peritoneal fluid ultimately reaches the blood serum, where it is detected as the CA-125 antigen (Bast et al. 1983). CA-125 has been the standard molecular marker of EOC malignancy for several decades due to its elevated serum levels in 80% of advanced stage EOC patients, but it is still considered to be an imperfect tool for early detection (Bast et al. 1983; Tuxen et al. 1995; Mai et al. 2011). MUC16 functions in EOC metastasis have been well described. The interaction between MUC16 and mesothelin, a protein present on the surface of mesothelial cells, has been extensively investigated and many studies have implicated CA-125:mesothelin binding in the early adhesive events of EOC metastasis (Rump et al. 2004; Gubbels et al. 2006; Kaneko et al. 2009; Chen et al. 2013). While several studies have reported that CA-125 shedding can be modulated by cell cycle functions (cells predominantly in S and G₂-M phase show reduced CA-125 shedding), tyrosine kinase inhibition, and interferon- γ (Marth et al. 1989, 2007; Zeimet et al. 1996), the precise impetus for MUC16 ectodomain shedding (and the corresponding increase in circulating CA-125), however, remains to be elucidated. Herein we present evidence to implicate MT1-MMP in this process.

Initial experiments to evaluate MUC16 expression on the surface of metastatic ovarian cancer tumor tissue indicated moderate to strong expression along tumor borders, irrespective of histotype. Interestingly, comparison with serial sections stained for the cell surface proteinase MT1-MMP revealed an inverse correlation between MT1-MMP and MUC16 immunoreactivity. These results were confirmed using ovarian cancer cell lines. OVCAR3 cells, which express high MUC16 levels, do not exhibit surface expression of MT1-MMP. Furthermore, when MUC16-expressing OVCA433 cells were engineered to overexpress MT1-MMP, surface expression of MUC16 was lost while mRNA expression remained unaffected. This was accompanied by enhanced soluble MUC16 ectodomain antigen in the conditioned medium. Incubation of these cells with a broad spectrum MMP inhibitor or expression of a catalytically inactive MT1-MMP mutant abrogated this effect.

Together these results support the hypothesis that MT1-MMP catalyzes MUC16 ectodomain shedding, releasing the CA-125 antigen from the cell surface.

We have previously demonstrated that expression of MT1-MMP can function to promote pro-metastatic behavior of EOC cells, including invasion of the interstitial collagen-rich sub-mesothelial matrix and promotion of proliferation in constrained three-dimensional collagen gels (Barbolina et al. 2007; Moss et al. 2009b). However, it may be predicted that sub-mesothelial collagen-invasive activity is secondary to an initial heterotypic cell-cell adhesive event occurring between the ovarian cancer cell and the mesothelial cell. MUC16 has been shown to interact strongly with mesothelin, with cell to mesothelin binding occurring via the N-linked glycoproteins of cell surface bound MUC16 (Gubbels et al. 2006). Hence, the interaction between mesothelial cell membrane-bound mesothelin and tumor cell surface MUC16 may represent the inaugural adhesive event prior to invasion. Indeed, results showing decreased adhesion of cells expressing catalytically active MT1-MMP to three-dimensional meso-mimetic cultures as well as to intact *ex vivo* peritoneal tissue explants support this hypothesis, revealing a loss of ovarian tumor cell:mesothelial cell adhesion in MT1-MMP expressing cells with reduced cell surface MUC16. Further support lies in the significantly increased adhesion of catalytically inactive MT1-MMP expressing cells to intact *ex vivo* peritoneal tissue. These data are highly suggestive of a role for MT1-MMP in potentiating MUC16 functionality in ovarian tumor cell attachment to mesothelin. Nevertheless, enhanced meso-mimetic invasion is observed in MT1-MMP-expressing OVCA433 cells, suggesting that though initial ovarian tumor cell:mesothelial cell interaction is dependent on MUC16 expression, additional molecules must be employed to engage the necessary heterotypic adhesion.

The role of integrins as mediators of ovarian cancer adhesion has been widely reported, with specific attention given to the interaction between $\beta 1$ integrin and fibronectin, expressed on ovarian cancer and mesothelial cells, respectively (Lessan et al. 1999; Strobel and Cannistra 1999; Casey et al. 2001; Ahmed et al. 2005; Desgrosellier and Cheresch 2010). Furthermore, our group has also demonstrated that ovarian cancer cells exhibit preferential adhesion to interstitial collagens in a $\beta 1$ integrin-dependent process (Moser et al. 1996; Ellerbroek et al. 2001). Interestingly, adhesion of OVCA433-MT cells (with low surface MUC16) to a mesothelial monolayer was inhibited by $\beta 1$ integrin function-blocking antibodies ($p=.001$) while adhesion of OVCA433-MT-E240A (high surface MUC16) remained unaffected ($p=.76$, data not shown). Together these data support the hypothesis that initial events in ovarian tumor cell:mesothelial cell adhesion are preferentially mediated by MUC16, transitioning to $\beta 1$ integrins either in the absence of MUC16 or to stabilize adhesive contacts. Future studies aimed at assessing $\beta 1$ integrin-mediated adhesive interactions, as well as identifying the downstream effectors of those interactions, may be instrumental towards elucidating specific molecular mechanisms involved in early ovarian tumor cell adhesive events.

We have previously demonstrated that expression of MT1-MMP is enhanced in experimentally generated ovarian cancer MCAs relative to single cells (Moss et al. 2009b). It has been reported that MCAs, or spheroids, isolated from human ovarian cancer ascites are less adhesive compared to individual ovarian carcinoma cells (Burlinson et al. 2004b). Burlinson and coworkers postulated that cells in spheroid form may favor homotypic

interactions over heterotypic interactions with mesothelium, and further suggest that adhesion complexes between MCAs and mesothelium are of lower affinity than those of single cells and are thus more susceptible to disruption by shear forces present in ascites fluid (Burleson et al. 2004a). Therefore, it is interesting to speculate that the MCA population in human ovarian cancer ascites represents a subpopulation of cells with relatively higher MT1-MMP and lower MUC16 compared to free floating single cells; however this remains to be investigated.

In summary, our data support a model wherein acquisition of catalytically active MT1-MMP expression in ovarian cancer cells induces MUC16 ectodomain shedding, resulting in increased soluble MUC16. Cells with decreased cell surface expression of MUC16 exhibit reduced adhesion to meso-mimetic cultures and to intact peritoneal explants. Nevertheless, MT1-MMP-expressing cells are more invasive through mesomimetic cultures comprised of mesothelial cells overlaying a 3-dimensional collagen matrix. These data support a model wherein initial interactions of ovarian tumor cells with peritoneal mesothelium may be facilitated by a lower affinity interaction between MUC16, which protrudes extensively from the cell surface, and mesothelin. Proteolytic clearing of MUC16, catalyzed by MT1-MMP, may then expose integrins for high affinity cell binding to peritoneal tissues, thereby anchoring metastatic lesions for subsequent proliferation within the collagen-rich sub-mesothelial matrix.

MATERIALS AND METHODS

Materials

MT1-MMP antibodies for immunofluorescence (IF) and/or immunohistochemistry (IHC) were purchased from Epitomics (Burlingame, Ca); CA-125 antibody for IHC was purchased from Dako (Carpinteria, Ca); and MUC1 and MUC16 antibodies for IF and flow cytometry were purchased from Abcam (Cambridge, Ma). Rat tail collagen type I was purchased from BD Biosciences (San Diego, CA). SYLGARD® 184 Silicone Elastomer Kit was purchased from Dow Corning (Midland, Mi). Cell culture media and additives were obtained from Invitrogen (Carlsbad, Ca) unless otherwise stated. Geneticin® (G418) was obtained from Life Technologies, Inc (Carlsbad, Ca). Galardin/Ilomastat (GM6001) and β 1 integrin functional inhibitor P5D2 was purchased from Millipore (Billerica, Ma). All chemicals were analytical grade.

Cell Culture

The ovarian cancer cell line OVCA433 was provided by Dr. Robert Bast, Jr. (MD Anderson Cancer Center, Houston, Tx), the ovarian adenocarcinoma cell line OVCAR3 was obtained from American Type Culture Collection (ATCC, Manassas, Va) and the human mesothelial cell line LP9 was obtained from Coriell Aging Cell Repository. All cells were maintained in a humidified incubator under standard conditions (37°C with 5% CO₂). OVCA433 cells were maintained in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 1mM non-essential amino acid, 50U/ml penicillin, and 50µg/ml streptomycin. OVCAR3 cells were maintained in RPMI 1640, 20% FBS, 1mM sodium pyruvate, 1mM nonessential amino acid, 2mM L-glutamine, 50U/ml penicillin, and

50µg/ml streptomycin. The LP9 cell line was maintained in 1:1 ratio Medium 199 and Ham's F12, supplemented with 15% FBS, 2mM glutamine, 1mM HEPES, 20mg/ml epidermal growth factor (R&D systems, Minneapolis, Mn), 0.4µg/ml hydrocortisone, 50U/ml penicillin, and 50µg/ml streptomycin. The transfection and generation of the stable cell lines OVCA433-MT and OVCA433-E240A have been previously described (Moss et al. 2009a). The E240A mutant of MT1-MMP represents a catalytically inactive mutation. Transfected cells were maintained in the parental OVCA433 media described above, with the addition of selection antibiotic Geneticin® (700µg/ml). Cells were routinely FACS-sorted every 3–5 passages with anti-MT1-MMP antibodies to insure continued cell surface expression using FACSaria III Cell Sorter (BD Biosciences, San Jose, Ca) and all experiments were performed with freshly sorted cell populations. Untransfected parental OVCA433 cells and OVCA433 cells transfected with empty vector were designated as "control"; both behaved identically in assays and were used interchangeably.

Immunohistochemistry

Immunohistochemical detection of antigen content in malignant ovarian tissue was performed by using tissue microarrays (US Biomax, Rockville, MD) containing 50 cores of ovarian adenocarcinoma. Slides were deparaffinized in xylene for 5 minutes (thrice) and soaked in absolute alcohol for 3 minutes. To inhibit endogenous peroxidase, slides were incubated for 30 minutes in 3.3% H₂O₂ in methanol. Antigen retrieval was accomplished by incubation in heated sodium citrate (10mM) for 1 hour. Slides were processed using VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, Ca). Nonspecific interactions were blocked by using normal horse serum for 30 minutes at room temperature. Primary antibodies were diluted in 0.01% PBS, pH 7.4 and incubated overnight at 4°C in a humidified chamber. Bound antibodies were detected by using biotinylated secondary antibody and streptavidin-conjugated HRP enzyme coupled with 3,3'-diaminobenzidine chromagen solution (BioGenex, San Ramon, Ca). Tissue was counterstained with hematoxylin and blued with saturated lithium carbonate solution. Staining was scored on a 1–3 scale where 1= no to low staining; 2=moderate staining; 3=strong staining.

Immunofluorescence

Cells were subcultured on glass coverslips, washed twice with ice-cold phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde in 0.12M sucrose in PBS for 10 minutes at room temperature. Cells were blocked with 5% normal goat serum in PBS for 1 hour at room temperature, incubated with primary antibody (1:100) in 1% normal goat serum in PBS for 1hr at 37°C, rinsed thrice for 5 minutes with PBS, and incubated with either Alexa-Fluor 488- or 594-conjugated secondary antibody (Invitrogen, Carlsbad, Ca) at a 1:500 dilution for 30 minutes at 37°C. After washing, cells were allowed to dry, mounted with VECTASHIELD Mounting Media with 4', 6-diamidino-2-phenylindole (DAPI) (Vector laboratories, Burlingame, Ca), and imaged an EVOS® FL digital inverted fluorescence microscope (Advanced Microscopy Group, Mill Creek, Wa).

Quantitative Real Time PCR (qPCR)

RNA was extracted from 10⁶ cells using RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. cDNA was synthesized from 1–5µg of total RNA using RT²

First Strand Kit (Qiagen). Real time PCR utilized StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). Conditions for amplification were as follows: an initial denaturation for 10 min at 95°C followed by 40 cycles of 95°C for 15s and 60°C for 30s using the iTaq SYBR Green Supermix (BioRad) as a fluorescent reporter. PCR primer specificity was determined via melting curves, where products were heated at 95°C, cooled to 65°C, and then slowly melted at 0.5°C/s up to 95°C. Primer sequences for MUC16 are as follows: 5'-TGC GGT GTC CTG GTG ACC-3', 3'-CAC CGG CAA GTT CCA GTC-5'. The comparative C_T method ($2^{-(C_{T \text{ sample}} - C_{T \text{ control}})}$) was used to determine average relative quantitation of MUC16. Results represent the average of triplicate experiments and show mean $\pm$ SEM. Student's t-test was used to determine p values (Sigmaplot).

FACS analysis

Subconfluent cells were harvested with 20mM EDTA and washed twice with PBS. (For studies utilizing inhibitor GM6001, subconfluent monolayers were incubated in 25μg/ml for 18–20 hours at 37°C). Cells (2×10^5) recovered in complete media for 30 minutes at 37°C, were centrifuged, washed, and immunostained using indicated antibody for 30 minutes at 4°C according to the manufacturer's instructions. Cells were stained with secondary antibody conjugated to Alexa Fluor 647 (1:500) for 30 minutes at 4°C, washed twice, and resuspended in 0.5ml PBS for analysis on a Beckman Coulter FC500 Flow Cytometer (Beckman Coulter, Inc, Indianapolis, In). Data was analyzed using FlowJo (TreeStar, Ashland, Or). Experiments were conducted in triplicate. Results are expressed as an average of the percentage of fluorescent units found in each treatment compared to secondary stained controls. Student's t-test was used to determine p values (Sigmaplot).

Enzyme-linked immunosorbent assay

Protein concentrations of CA-125 secreted in the conditioned media of freshly sorted cells plated for 48 hours (up to confluence) were measured using a commercially available sandwich ELISA kit (Sigma, St. Louis, Mo), using 96-well plates coated with antibody specific for human CA-125. After incubation, media is collected, centrifuged gently to remove cellular debris, and pipetted into wells where CA-125 present in the sample is bound by the immobilized antibody. Biotinylated detection antibody and HRP-conjugated streptavidin were used for detection of bound CA-125. Experiments were assayed in duplicate and collected at least twice to verify the result. Absorbance readings were made at 450nm and were acquired on a Molecular Devices Spectramax microtiter plate reader (Sunnyvale, Ca). CA-125 levels were determined by interpolation from a standard curve.

Mesothelial cell adhesion assay

Tissue culture wells were coated with 10μg/ml collagen type I in sodium carbonate, pH 9.6 overnight at 4°C, washed with PBS, and air dried. LP9 cells were seeded and grown for 48 hours to form a tightly woven monolayer. Cancer cells were labeled with CellTracker™ Green, (5-Chloromethylfluorescein Diacetate, Life Technologies Inc, Carlsbad, Ca) for 30 minutes at 37°C. The live mesothelial monolayer was washed twice with PBS, seeded with cells as indicated, and allowed to adhere for 35 minutes. Wells were then washed with PBS to remove non-adherent cells, fixed, and fluorescent cells enumerated. Assays were

performed in triplicate and five fields/well were counted. Results show mean $\pm$ SEM. Student's t-test was used to determine p values (Sigmaplot).

Meso-mimetic invasion assay

A three-dimensional collagen type I matrix was prepared (previously described, (Moss et al. 2009b)) with or without fibroblasts atop an 8 μ m microporous membrane within a transwell insert (BD Biosciences, San Diego, Ca) and overlaid with LP9 cells (Lengyel et al. 2013). The LP9 were allowed to grow to confluence, forming a tight monolayer. CellTracker™ Green-labelled OVCA433 cells were seeded atop the live monolayer and the co-culture was incubated at 37°C for 48 hours in a 1:1 ratio of complete media for each cell type. Migrated cells passing through the 3-dimensional culture and the 8 μ m pore filter were fixed and stained with Diff-Quik (Fisher Scientific, Pittsburgh, PA) and enumerated. All experiments were completed in triplicate and five fields/well were counted. Results show mean $\pm$ SEM. Student's t-test was used to determine p values (Sigmaplot).

Adhesion to a peritoneal explant

Peritoneal tissues from female c57bl/6 mice were excised, rinsed twice, and submerged in PBS. While submerged, tissues were uniformly trimmed to a 2.0 $\times$ 2.0mm square and pinned to a SYLGARD® 184 silicone coated dish. Cells to be assayed were labeled with CellTracker™ Green, trypsinized, diluted to 200,000cells/ml, and 2.5ml of cell suspension was added to tissue and incubated for 2 hours at 37°C. After incubation, the tissue was washed vigorously five times, removed from the silicone bed, and mounted onto a glass coverslip for imaging (EVOS® FL) and enumeration. The number of cells/5 fields was counted and the assay was performed in triplicate. Results show mean $\pm$ SEM. Student's t-test was used to determine p values (Sigmaplot). Alternatively, tissues were processed for scanning electron microscopy, as described previously (Barbolina et al. 2013). Briefly, post-assay tissues were washed, fixed, dehydrated, and dried. Once dried, samples were placed on carbon stubs, subjected to Flash-Dry™ silver paint, coated with platinum, and examined using a Magellan 400 scanning electron microscope (FEI, Hillsboro, Or).

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Figure 1.

Expression of MT1-MMP and MUC16 in ovarian cancer cells and tissues. Microarrayed cores of ovarian adenocarcinoma were subjected to immunohistochemical analyses for MT1-MMP (A–C) or MUC16 (D–F) as described in Methods. Examples of low (A,F), moderate (B,E) and high (C,D) staining are shown.

Figure 2.
Sub-confluent monolayers of OVCA433, OVCA433-MT, OVCA433-E240A or OVCAR3 cells (as indicated) were processed for immunofluorescence. Staining for MT1-MMP (green - A, C, E, G) or MUC16 (red - B, D, F, H) as indicated.

Figure 3.

MT1-MMP overexpression is associated with decreased cell surface expression of MUC16. (A) Quantitative real time PCR analysis of MUC16 levels. No significant differences in MUC16 expression levels are observed. (B) Flow cytometric analysis of surface MUC16. (C) Quantitation of shed MUC16 ectodomain as determined by ELISA in 48 h conditioned culture media. Red bars – OVCA433; green bars – OVCA433-MT1-MMP; yellow bars – OVCA433-MT1-MMP cultured with the broad spectrum MMP inhibitor GM6001; blue bars – OVCA433-MT1-MMP-E240A mutant.

Figure 4.

Expression of MT1-MMP alters tumor cell adhesion to and invasion through a live mesothelial monolayer. **(A)** Schematic representation of adhesion assay depicting representative image of ovarian tumor cells (green) attached to a confluent mesothelial cell (LP9) monolayer. **(B)** CMFDA-labeled cells were allowed to adhere to a confluent monolayer of LP9 mesothelial cells for 30 min prior to washing to remove unbound cells and quantitation of adherent fluorescent cells. Results are quantified as a percentage of total cells seeded. **(C)** Schematic representation of meso-mimetic invasion assay. **(D)** CMFDA-labeled cells were seeded atop a meso-mimetic culture comprised of collagen embedded fibroblasts overlaid with a layer of LP9 mesothelial cells in a Boyden chamber containing an 8 μ m pore filter. Following incubation for 48 h, cells penetrating the meso-mimetic through the pores to the underside of the filter were enumerated. Results depict the ratio of migrated cells to total cells seeded. Red bars – OVCA433; green bars – OVCA433-MT1-MMP; yellow bars – OVCA433-MT1-MMP cultured with the broad spectrum MMP inhibitor GM6001; blue bars – OVCA433-MT1-MMP-E240A mutant.

Figure 5.

Expression of MT1-MMP affects cell-to-mesothelial adhesion in an *ex vivo* peritoneal explant. (A–C) Depiction of assay. (A) Ovarian cancer cells were fluorescently labeled with CMFDA. (B) An excised explant from the peritoneum of a female mouse was pinned, mesothelium-side up, to a tissue culture dish containing an optically clear silastic resin. Tumor cells were allowed to adhere for 2 h prior to washing to remove unlabeled cells. (C) Representative scanning electron micrograph showing tumor cells (round) adherent to mesothelial monolayer. (D–F) Representative images of fluorescently labeled ovarian tumor

cells attached to explant. (G) Quantitation of adhesion of OVCA433, OVCA433-MT, or OVCA433-EA to murine peritoneal tissue explant. Results are expressed as the relative number of cells per area. Red bars – OVCA433; green bars – OVCA433-MT1-MMP; blue bars – OVCA433-MT1-MMP-E240A mutant.