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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**

**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, whereas 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 meso-mimetic cultures and intact *ex vivo* peritoneal tissue explants was observed. Nevertheless, meso-mimetic 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 comprises a single layer of nondistinguished 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,
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 (MCAs), 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 sub-mesothelial 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 Da). This heavily glycosylated mucin, containing both O- 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 physiological 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 neither detected in normal OSE nor in benign ovarian tumors but is widely expressed in ovarian carcinomas of all histological 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 three-dimensional (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 (Figure 1A–C) or MUC16 (Figure 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 (Figure 1C) exhibit low MUC16 (Figure 1F) and tissues with elevated MUC16 (Figure 1D) do not stain positively for MT1-MMP (Figure 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 (Figure 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 (Figure 2A and B). Similar results were obtained with parental OVCA433 cells.
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 the Materials and methods section. Examples of low (A and F), moderate (B and E), and high (C and D) staining are 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 h, after which the conditioned media were assessed for human CA-125 using a commercially available enzyme-linked immunosorbent assay (ELISA). Although quantifiable CA-125 was found in all samples (Figure 3C), the concentration of CA-125 found in conditioned medium from OVCA433-MT cells was significantly increased compared with 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 (Figure 4A). Whereas parental OVCA433 cells adhered avidly to mesothelial cells (Figure 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 the penetration of a more physiologically relevant cell-matrix context, the ability to invade 3D meso-mimetic cultures cultured atop porous membranes in a transwell was evaluated (Figure 4C; Lengyel et al., 2013). Expression of MT1-MMP significantly enhanced meso-mimetic invasion (Figure 4D), whereas 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 (Figure 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 (Figure 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 intraperitoneally through adhesion to and localized invasion of peritoneal mesothelium to anchor secondary lesions. Over 70% of EOC are diagnosed with intraperitoneal metastasis, when 5-year survival rates are <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). Although several studies have reported that CA-125 shedding can be modulated by cell cycle functions (cells predominantly in the S and the 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 OVAR433 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 3D collagen gels (Barbolina et al., 2007; Moss et al., 2009b). However, it may be predicted

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**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 OVAR433, OVAR433-MT, or OVAR433-EA to murine peritoneal tissue explant. Results are expressed as the relative number of cells per area. Red bars, OVAR433; green bars, OVAR433-MT1-MMP; blue bars, OVAR433-MT1-MMP-E240A mutant.
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 3D 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 although 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 β1-integrin and fibronectin, expressed on ovarian cancer and mesothelial cells, respectively (Lessa et al., 1999; Strobel and Cannistra, 1999; Casey et al., 2001; Ahmed et al., 2005; Desgrois and Cheresh, 2010). Furthermore, our group has also demonstrated that ovarian cancer cells exhibit preferential adhesion to interstitial collagens in a β1-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 β1-integrin function-blocking antibodies (p=0.001), whereas adhesion of OVCA433-MT-E240A (high surface MUC16) remained unaffected (p=0.76, 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 β1-integrins either in the absence of MUC16 or to stabilize adhesive contacts. Future studies aimed at assessing β1-integrin-mediated adhesive interactions as well as identifying the downstream effectors of those interactions may be instrumental toward 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). MCAs, or spheroids, isolated from human ovarian cancer ascites are less adhesive compared with individual ovarian carcinoma cells (Burleson et al., 2004b). Burleson et al. (2004a) postulated that cells in spheroid form may favor homotypic interactions over heterotypic interactions with mesothelium, suggesting 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. 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 with 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 meso-mimetic cultures comprising mesothelial cells overlaying a 3D 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 mesothelium. 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, USA); CA-125 antibody for IHC was purchased from Dako (Carpinteria, CA, USA); and MUC1 and MUC16 antibodies for IF and flow cytometry were purchased from Abcam (Cambridge, MA, USA). Rat-tail collagen type I was purchased from BD Biosciences (San Diego, CA, USA). SYLGARD® 184 Silicone Elastomer Kit was purchased from Dow Corning (Midland, MI, USA). Cell culture media and additives were obtained from Invitrogen (Carlsbad, CA, USA) unless otherwise stated. Geneticin® (G418) was obtained from Life Technologies (Carlsbad, CA, USA). Galardin/Ilomastat (GM6001) and β1-integrin...
functional inhibitor PSD2 were purchased from Millipore (Billerica, MA, USA). 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, USA); the ovarian adenocarcinoma cell line OVCAR3 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA); and the human mesothelial cell line LP9 was obtained from Coriell Aging Cell Repos-itory. All cells were maintained in a humidified incubator under standard conditions (37°C with 5% CO₂). OVCAR3 cells were maintained in RPMI 1640, 20% FBS, 1 mm sodium pyruvate, 50 μg/ml streptomycin. OVCAR3 cells were maintained in RPMI 1640, 20% FBS, 1 mm sodium pyruvate, 50 μg/ml streptomycin. The LP9 cell line was maintained in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS), 1 mm sodium pyruvate, 1 mm non-essential amino acid, 50 U/ml penicillin, and 50 μg/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 with primary antibody (1:100) in 1% normal goat serum in PBS for 1 h at room temperature, incubated with either Alexa-Fluor 488- or 594-conjugated secondary antibody (Invitrogen) at a 1:500 dilution for 30 min at 37°C. After washing, cells were allowed to dry, mounted with VECTASHIELD Mounting Media with 4′,6-diamidino-2-phenylindole (Vector Laboratories), and imaged on an EVOS® FL digital inverted fluorescence microscope (Advanced Microscopy Group, Mill Creek, WA, USA).

Quantitative real-time PCR

RNA was extracted from 10⁵ cells using RNeasy Mini Kit (Qiagen) in accordance with the manufacturer’s instructions. Complementary DNA was synthesized from 1 to 5 μg 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 15 s and 60°C for 30 s 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 95°C, rinsed thrice for 5 min with PBS, and incubated with either Alexa-Fluor 488- or 594-conjugated secondary antibody (Invitrogen) at a 1:500 dilution for 30 min at 37°C. After washing, cells were allowed to dry, mounted with VECTASHIELD Mounting Media with 4′,6-diamidino-2-phenylindole (Vector Laboratories), and imaged on an EVOS® FL digital inverted fluorescence microscope (Advanced Microscopy Group, Mill Creek, WA, USA).

Immunohistochemistry

Immunohistochemical detection of antigen content in malignant ovarian tissue was performed using tissue microarrays (US Biomax, Rockville, MD, USA) containing 50 cores of ovarian adenocarcinoma. Slides were de-paraffinized in xylene for 5 min (thrice) and soaked in absolute alcohol for 3 min. To inhibit endogenous peroxidase, slides were incubated for 30 min in 3.3% H₂O₂ in methanol. Antigen slides were de-paraffinized in xylene for 5 min (thrice) and soaked in absolute alcohol for 3 min. To inhibit endogenous peroxidase, slides were incubated for 30 min in 3.3% H₂O₂ in methanol. Antigen retrieval was accomplished by incubation in heated sodium citrate (10 mM) for 1 h. Slides were processed using VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Nonspecific interactions were blocked using normal horse serum for 1 h at room temperature. Primary antibodies were diluted in 0.01% phosphate-buffered saline (PBS), pH 7.4, and incubated overnight at 4°C in a humidified chamber. Bound antibodies were detected using biotinylated secondary antibody and streptavidin-conjugated horseradish peroxidase (HRP) enzyme coupled with 3,3′-diaminobenzidine chromagen solution (BioGenex, San Ramon, CA, USA). 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.

FACS analysis

Sub-confluent cells were harvested with 20 mM EDTA and washed twice with PBS. (For studies utilizing inhibitor GM6001, sub-confluent monolayers were incubated in 25 μg/ml for 18–20 h at 37°C.) Cells (2×10⁶) recovered in complete media for 30 min at 37°C were centrifuged, washed, and immunostained using indicated antibody for 30 min at 4°C according to the manufacturer’s instructions. Cells were stained with secondary antibody conjugated to Alexa Fluor 647 (1:500) for 30 min at 4°C, washed twice, and resuspended in 0.5 ml PBS for analysis on a Beckman Coulter FC500 Flow Cytometer (Beckman Coulter, Indianapolis, IN, USA). Data were analyzed using FlowJo (TreeStar, Ashland, OR, USA). Experiments were conducted in triplicate. Results are expressed as an average of the percentage of fluorescent units found in each treatment compared with 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 h (up to confluence) were measured
with a commercially available sandwich ELISA kit (Sigma, St. Louis, MO, USA), 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 450 nm and were acquired on a Molecular Devices Spectramax microtiter plate reader (Sunnyvale, CA, USA). CA-125 levels were determined by interpolation from a standard curve.

Mesothelial cell adhesion assay

Tissue culture wells were coated with 10 µg/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 h to form a tightly woven monolayer. Cancer cells were labeled with CellTracker™ Green (5-chloromethylfluorescein diacetate; Life Technologies) for 30 min at 37°C. The live mesothelial monolayer was washed twice with PBS, seeded with cells as indicated, and allowed to adhere for 35 min. Wells were then washed with PBS to remove non-adherent cells, fixed, and fluorescent cells enumerated. Assays were performed in triplicate, and 5 fields per well were counted. Results show mean ± SE. Student’s t-test was used to determine p-values (Sigmaplot).

Meso-mimetic invasion assay

A 3D collagen type I matrix was prepared (previously described; Moss et al., 2009b) with or without fibroblasts atop an 8-µm microporous membrane within a transwell insert (BD Biosciences) and overlaid with LP9 cells (Lengyel et al., 2013). The LP9 were allowed to grow to confluence, forming a tight monolayer. CellTracker™ Green-labeled OVCAR3 cells were seeded atop the live monolayer, and the co-culture was incubated at 37°C for 48 h in a 1:1 ratio of complete media for each cell type. Migrated cells passing through the 3D culture and the 8-µm pore filter were fixed and stained with Diff-Quik (Fisher Scientific, Pittsburgh, PA, USA) and enumerated. All experiments were completed in triplicate, and 5 fields per well were counted. Results show mean ± SE. 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 submersed in PBS. While submersed, tissues were uniformly trimmed to a 2.0×2.0-mm square and pinned to a SYLGARD® 184 silicone-coated dish. Cells to be assayed were labeled with CellTracker™ Green, trypsinized, and diluted to 200 000 cells/ml, and 2.5 ml of cell suspension was added to tissue and incubated for 2 h 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 per five fields was counted, and the assay was performed in triplicate. Results show mean ± SE. 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, USA).

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