

# Ovarian Cancer Cell Detachment and Multicellular Aggregate Formation Are Regulated by Membrane Type 1 Matrix Metalloproteinase: A Potential Role in I.p. Metastatic Dissemination

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## Abstract

An early event in the metastasis of epithelial ovarian carcinoma is shedding of cells from the primary tumor into the peritoneal cavity followed by diffuse i.p. seeding of secondary lesions. Anchorage-independent metastatic cells are present as both single cells and multicellular aggregates (MCA), the latter of which adhere to and disaggregate on human mesothelial cell monolayers, subsequently forming invasive foci. Although this unique metastatic mechanism presents a distinct set of therapeutic challenges, factors that regulate MCA formation and dissemination have not been extensively evaluated. Proteolytic activity is important at multiple stages in i.p. metastasis, catalyzing migration through the mesothelial monolayer and invasion of the collagen-rich submesothelial matrix to anchor secondary lesions, and acquisition of membrane type 1 matrix metalloproteinase (MT1-MMP; MMP-14) expression promotes a collagen-invasive phenotype in ovarian carcinoma. MT1-MMP is regulated posttranslationally through multiple mechanisms including phosphorylation of its cytoplasmic tail, and the current data using ovarian cancer cells expressing wild-type, phosphomimetic (T567E-MT1-MMP), and phosphodeficient (T567A-MT1-MMP) MT1-MMP show that MT1-MMP promotes MCA formation. Confluent T567E-MT1-MMP-expressing cells exhibit rapid detachment kinetics, spontaneous release as cell-cell adherent sheets concomitant with MT1-MMP-catalyzed  $\alpha_3$  integrin ectodomain shedding, and robust MCA formation. Expansive growth within three-dimensional collagen gels is also MT1-MMP dependent, with T567E-MT1-MMP-expressing cells exhibiting multiple collagen invasive foci. Analysis of human ovarian tumors shows elevated MT1-MMP in metastases relative to paired primary tumors. These data suggest that MT1-MMP activity may be key to ovarian carcinoma metastatic success by promoting both formation and dissemination of MCAs. [Cancer Res 2009;69(17):7121–9]

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-08-4151

## Introduction

Ovarian cancer is the leading cause of death from gynecologic malignancy due primarily to complications of metastasis (1). Unlike most carcinomas that rely on the vasculature for metastasis, an early event in ovarian cancer dissemination is shedding of cells from the primary tumor into the peritoneal cavity followed by diffuse “seeding” of the peritoneal cavity (2). This unique metastatic mechanism presents a distinct set of therapeutic challenges. Accumulation of malignant ascites is widely associated with advanced ovarian carcinoma (3) and it is hypothesized that ascites augment progression by facilitating the spread of cancer cells throughout the peritoneal cavity (4). Anchorage-independent metastatic cells are present as both single cells and multicellular aggregates (MCA) that survive in suspension (5). The factors that regulate MCA formation have not been extensively evaluated; however, evidence suggests that MCAs have a functional role in metastatic progression. MCAs adhere to and disaggregate on human mesothelial cell monolayers, subsequently forming invasive foci (6, 7). Transplanted ovarian cancer MCAs form murine xenografts with the same histopathology as the primary tumor and can be serially propagated *in vivo* (8, 9). Further, sphere-forming ovarian cancer-initiating cells are significantly more tumorigenic in xenograft models, further showing that the MCA population is a key target for antimetastatic therapy (10).

Proteolytic activity is important at multiple stages in i.p. metastasis, including localized proteinase-driven migration through the mesothelial monolayer and invasion of the collagen-rich submesothelial matrix to anchor secondary lesions (11, 12). Invasion of collagenous matrices by ovarian cancer cells requires membrane type 1 matrix metalloproteinase (MT1-MMP; MMP-14; refs. 13–15), a transmembrane collagenase that is not detected in normal ovarian surface epithelium or in benign ovarian tumors, but is widely expressed in ovarian carcinomas of all histotypes (15–20). Acquisition of MT1-MMP expression promotes cell migration, extracellular matrix invasion, and growth within restricted three-dimensional matrices (21–23).

Because MT1-MMP is central to a variety of biological processes, proteolytic activity is stringently controlled. MT1-MMP is internalized from the cell surface through a mechanism involving the cytoplasmic domain (24, 25) and cytoplasmic tail truncation restricts MT1-MMP to the plasma membrane. The cytoplasmic domain of MT1-MMP has three potential phosphorylation sites: T<sup>567</sup>, Y<sup>573</sup>, and S<sup>577</sup>. Recent work indicates that MT1-MMP can be phosphorylated at T<sup>567</sup> and Y<sup>573</sup> (26–28). T<sup>567</sup> is localized within the sequence R<sup>563</sup>RHG<sup>T567</sup>PRRLLYCQRSLLDKV<sup>582</sup> that has homology with the consensus sequence for protein kinase C (TXR) and

extracellular signal-regulated kinase 1/2 (*XTP*; ref. 29). To examine the potential effect of T<sup>567</sup> phosphorylation in the unique metastatic mechanism of ovarian carcinoma, the properties of cells expressing wild-type MT1-MMP, a phosphomimetic mutant (T567E-MT1-MMP), or a phosphodeficient mutant (T567A-MT1-MMP) were evaluated. Acquisition of MT1-MMP catalytic activity promotes rapid cell-matrix detachment kinetics concomitant with  $\alpha_3$  integrin ectodomain shedding, enhanced MCA formation, and expansive growth in three-dimensional collagen. This prometastatic phenotype was intensified in the phosphomimetic mutant T567E-MT1-MMP, suggesting that phosphorylation of the MT1-MMP cytoplasmic tail may regulate i.p. metastatic dissemination.

## Materials and Methods

**Materials.** DOV13 and OVCA433 cells were provided by Dr. R. Bast. Anti-FLAG M2, anti-MT1-MMP (M3927), peroxidase-conjugated secondary antibodies, and protein G-Sepharose beads were from Sigma. SuperSignal enhanced chemiluminescence reagents were purchased from Pierce. Tissue inhibitor of metalloproteinase-2 (TIMP-2) was provided by Dr. R. Fridman. Rat tail collagen type I, human type IV collagen, and human fibronectin were purchased from BD Biosciences. Mouse anti-human integrin  $\alpha_3$  (AMB1952Z and MAB2056) was purchased from Chemicon. Centriprep was purchased from Millipore.

**DNA constructs and generation of stable cell lines.** The human MT1-MMP cDNA with COOH-terminal FLAG tag (DYKDDDDK) was provided by Dr. D. Pei. Subsequently, the T567A, T567E, and E240A point mutations were generated using QuikChange (Stratagene). Inserts were sequenced to verify mutation. Transfection of cells was done using FuGENE 6 (Roche) and stable cell lines were generated using G418 selection. Clones were routinely fluorescence-activated cell sorting sorted (every 3-5 passages) using anti-FLAG monoclonal M2 and pooled clones with similar expression levels were used for analyses. Untransfected parental OVCA433 cells ("control") and OVCA433 cells transfected with empty vector ("vector" or "vec") behaved identically in all assays and have been used interchangeably.

**Western blotting.** Cells were collected with lysis buffer [1% Triton X-100, 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 45 mmol/L EDTA, 0.1% SDS, 20 mmol/L NaF, 10 mmol/L Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>] and protein concentration was analyzed using the Bio-Rad D<sub>C</sub> detection kit. Cell lysates (50  $\mu$ g) were electrophoresed on 9% SDS-PAGE, transferred to polyvinylidene difluoride, and blocked with 3% bovine serum albumin (BSA) in 50 mmol/L Trizma, 300 mmol/L NaCl, and 0.2% Tween 20 (TBST). Membranes were incubated (1 h, room temperature) with FLAG M2 monoclonal (1:1,000) or anti-MMP-14 in 3% BSA/TBST. Immunoreactive bands were visualized with a peroxidase-conjugated anti-rabbit IgG (1:4,000 in 3% BSA/TBST) and enhanced chemiluminescence.

To detect integrin  $\alpha_3$  in conditioned medium, 10<sup>6</sup> cells were serum starved overnight  $\pm$  the broad-spectrum MMP inhibitor GM6001. Conditioned medium was collected, concentrated, then electrophoresed on 9% SDS-PAGE, transferred to polyvinylidene difluoride, and blocked with 3% BSA/TBST. Membranes were incubated (1 h, room temperature) with anti-integrin  $\alpha_3$  (1:1,000; MAB1952Z) in 3% BSA/TBST. Immunoreactive bands were visualized with a peroxidase-conjugated anti-mouse IgG (1:4,000 in 3% BSA/TBST) and enhanced chemiluminescence. For immunoprecipitation, 30  $\mu$ L protein G beads were added to conditioned medium (6 mL) and rotated for 2 h at 4°C. Anti-integrin  $\alpha_3$  antibody (5  $\mu$ g; MAB2056) was added and samples were rotated overnight at 4°C followed by addition of protein G beads (35  $\mu$ L) for 2 h. Samples were centrifuged, washed with lysis buffer, and electrophoresed as described.

**Flow cytometric analysis.** Cells (10<sup>6</sup>) were treated with either monoclonal anti-FLAG for MT1-MMP detection (1:100) or monoclonal anti-integrin  $\alpha_3$  (1:100; MAB1952Z) for 30 min at 4°C. Cells were stained with secondary antibody conjugated to Alexa Fluor 488 (1:200) for 30 min at 4°C, washed twice with PBS, and resuspended in 0.7 mL PBS for analysis with Summit Software 4.3 on a Beckman Coulter fluorescence-activated cell

sorter. Results are expressed as average of mean fluorescence units of experiments conducted in triplicate.

**MCA formation.** MCAs were generated using a modification of the hanging drop method as described previously (30). MCA formation was monitored after 24 to 48 h.

**RNA extraction, cDNA synthesis, and real-time reverse transcription-PCR.** RNA was extracted from 10<sup>6</sup> cells using the SV Total RNA Isolation System (Promega). cDNA was synthesized from 5 to 10  $\mu$ g total RNA using iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR used SYBR Green chemistry and the 7500 ABI Prism (Applied Biosystems). Reaction setup, normalization, and primers were used as described previously and fold changes were quantified as 2<sup>-( $\Delta$ Ct sample -  $\Delta$ Ct control)</sup> (15).

**Quantitation of cell-matrix detachment.** Cells (10<sup>6</sup>) were seeded onto 60 mm dishes and allowed to adhere overnight. Adherent cells were subjected to controlled trypsinization (0.25% trypsin/2.21 mmol/L EDTA in HBSS without sodium bicarbonate, calcium, and magnesium) and both adherent and detached cells were quantified. Experiments were repeated three to five times.

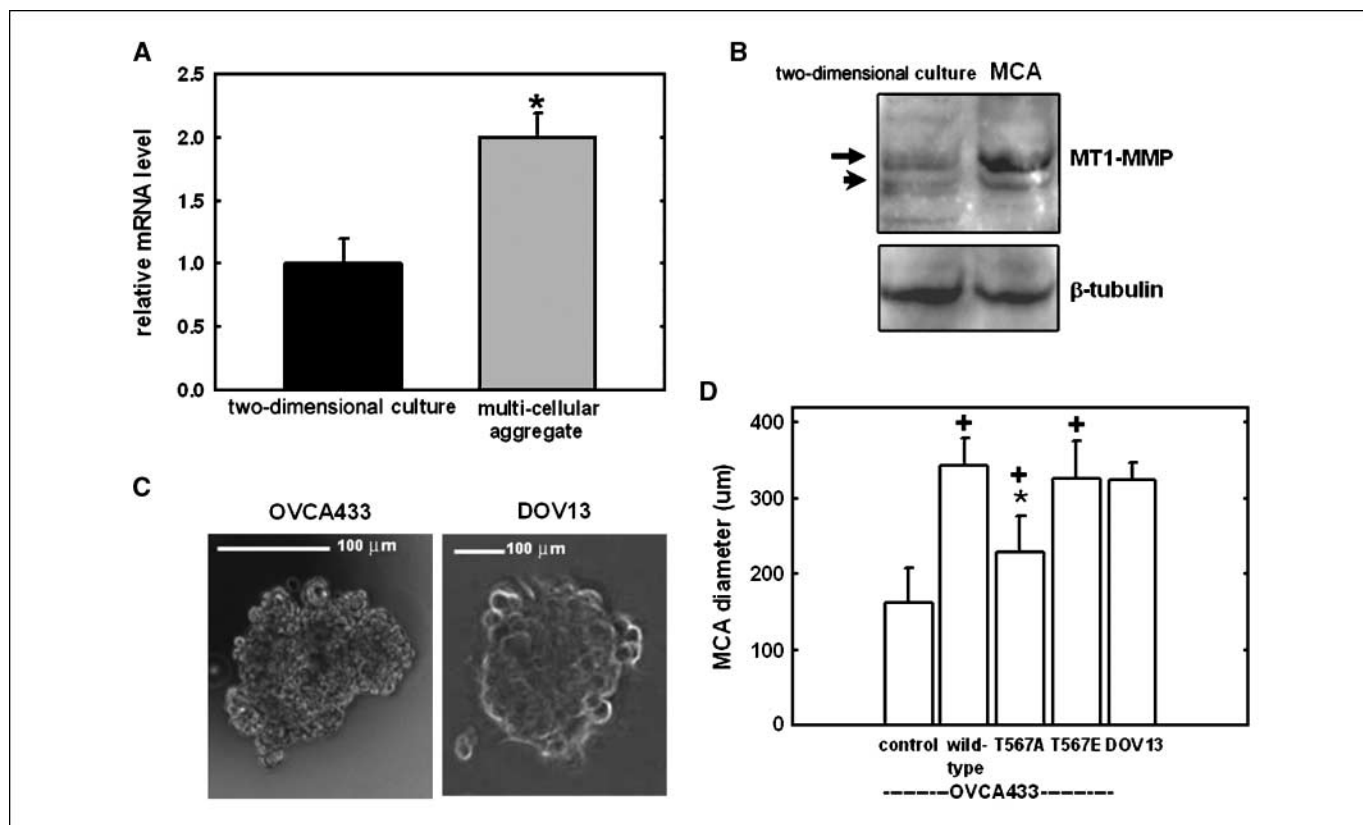
**Adhesion assays.** Chambers were coated with 10  $\mu$ g/mL type I collagen, type IV collagen, or fibronectin in PBS for 4 h at 37°C, blocked with 3% BSA in MEM for 1 h at 37°C, washed with PBS, and air dried. Cells were seeded at 10<sup>5</sup> per well, allowed to adhere for 75 min (determined empirically based on a time course of 0-2 h), washed with PBS to remove nonadherent cells, fixed, stained using DiffQuick, and enumerated. Assays were done in triplicate.

**Three-dimensional collagen culture.** Three-dimensional cultures were prepared as described (31) by diluting type I rat tail collagen with 10 $\times$  MEM to 1.5 mg/mL. Cells (10<sup>4</sup>) were added to the collagen mixture before solidification  $\pm$  TIMP-2 (5  $\mu$ g/mL). In additional controls, cells were added atop solidified gels.

**Immunohistochemistry.** Immunohistochemical analysis was done retrospectively on tumor tissue microarrays prepared with institutional review board approval by the Pathology Core Facility of Northwestern University. Tissue microarrays included 17 paired primary and metastatic ovarian cancer tissues obtained during the same surgical procedure from patients who were not treated against stage III and IV ovarian cancer before the operation (15 serous and 2 endometrioid). Immunohistochemical staining used antibody to MT1-MMP (clone RB1544B; 1:100 dilution; Neomarkers; ref. 22) with breast carcinoma tissue as a positive control. Scoring was assigned according to the average overall intensity of the staining: 0, no staining; 1, fine granular staining; 2, somewhat coarse staining but less than positive control tissue (breast carcinoma); and 3, very coarse staining, similar to or greater than positive control tissue. Staining <10% of tumor cells, regardless of intensity, was considered negative. Staining of between 10% and 75% of tumor cells was considered focal positive. Staining of >75% of tumor cells was considered diffuse positive.

## Results

**Expression of MT1-MMP in ovarian cancer cells.** Based on previous studies showing that phosphorylation of cytoplasmic tail residues in MT1-MMP can modify cell behavior (26-28), MT1-MMP mutants were generated in which T<sup>567</sup> was mutated to glutamic acid (T567E) to mimic constitutive phosphorylation or alanine (T567A) to represent a phosphodeficient mutation. Constructs were transfected into OVCA433 cells chosen because of the lack of endogenous MT1-MMP expression. Analysis of MT1-MMP in whole-cell lysates obtained from wild-type and phosphomutant cell lines showed equivalent expression of wild-type and mutant MT1-MMP (Supplementary Fig. S1A). Relative to cells expressing wild-type MT1-MMP, gelatin zymography indicated no significant change in proMMP-2 activation by T567A-MT1-MMP or T567E-MT1-MMP mutant cell lines (refs. 16, 21, 32-35; Supplementary Fig. S1B). The cytoplasmic tail alters the surface presentation of active MT1-MMP by regulating its internalization from the cell



**Figure 1.** MT1-MMP expression in MCAs. *A*, expression of MT1-MMP RNA in DOV13 MCAs or 2D cultures. Real time RT-PCR was performed to detect RNA expression. Shown is the average of three independent experiments  $\pm$  standard deviation. (\*,  $P < 0.05$ ). *B*, expression of MT1-MMP protein. Western blot was performed to detect relative MT1-MMP protein levels with  $\beta$ -tubulin as a loading control. Arrow, 55 kDa active MT1-MMP; arrowhead, 43 kDa MT1-MMP autolysis product. *C*, representative MCA formed by OVCA 433 or DOV13 cells. *D*, expression of MT1-MMP augments MCA formation. Cells were cultured for MCA formation using the hanging drop method and measured using a Zeiss Axiovert Imaging Software. Data shown are expressed in  $\mu\text{m}$ , and represent the mean diameter measurement of  $n = 10$  MCAs. +,  $P < 0.05$ , relative to vector-transfected cells; \*,  $P < 0.05$ , relative to cells expressing wild type MT1-MMP. Untransfected DOV13 cells, that express similar levels of endogenous MT1-MMP, are shown for comparison.

surface (24, 25), the functional consequences of which may affect net proteolytic activity. Because the specific sequence(s) in the cytoplasmic tail responsible for modulating internalization have yet to be delineated, the effect of T<sup>567</sup> mutation on MT1-MMP surface presentation was evaluated. Relative to cells expressing wild-type, T567A-MT1-MMP<sup>-</sup>, and T567E-MT1-MMP<sup>-</sup> expressing cells exhibit similar basal levels of surface expression (Supplementary Fig. S1C). Further, expression levels of exogenous MT1-MMP in OVCA433 stable transfectants are similar to endogenous MT1-MMP levels in DOV13 cells (Supplementary Fig. S1D and E). Potential changes in the relative distribution of MT1-MMP mutants within specific membrane microdomains or in rates of internalization in response to specific stimuli were not evaluated.

**MT1-MMP promotes spontaneous detachment and MCA formation.** Initial dissemination of ovarian cancer involves exfoliation of cells from the primary ovarian tumor into the peritoneal cavity as matrix-detached single cells and MCAs (5, 6). Malignant ascites accumulates in advanced ovarian carcinoma and contains a population of nonadherent MCAs ranging in size from 30 to 200  $\mu\text{m}$  (36). These cells survive anoikis (37) and proliferate as a free-floating population of highly neoplastic cells (38); however, molecular mechanisms regulating the genesis of MCAs have not been extensively evaluated. Exploratory cDNA microarray analysis of changes in gene expression induced by MCA culture in DOV13 cells, which express endogenous MT1-MMP (13, 15), identified

MT1-MMP as 1 of <100 genes significantly up-regulated in MCA culture.<sup>6</sup> Validation using quantitative PCR and Western blotting confirmed these results, showing a 2.1-fold increase in MT1-MMP in MCAs relative to two-dimensional cultures (Fig. 1A and B). To evaluate the effect of acquisition of MT1-MMP expression on MCA formation, OVCA433 cells expressing wild-type or mutant MT1-MMP were evaluated relative to DOV13 using the hanging drop method (30) to generate MCAs *in vitro* (Fig. 1C). Cells expressing MT1-MMP formed larger diameter MCAs relative to untransfected control cells (Fig. 1D). Interestingly, smaller MCAs were formed by cells expressing the phosphodeficient T567A-MT1-MMP mutant relative to those expressing wild-type or T567E phosphomimetic mutant MT1-MMP, suggesting that phosphorylation at this site may modulate spheroid size and overall growth.

Although MCAs are prevalent in ascites obtained from women with ovarian carcinoma (6, 7, 36, 39), it is not known whether cellular aggregation occurs before or following detachment of metastatic cells from the primary tumor. Microscopic examination of cell cultures expressing T567E-MT1-MMP revealed a striking phenotype, showing spontaneous detachment as cell-cell adherent MCAs (Fig. 2A). To further characterize this phenotype, relative adhesive strength was evaluated using a controlled trypsinization

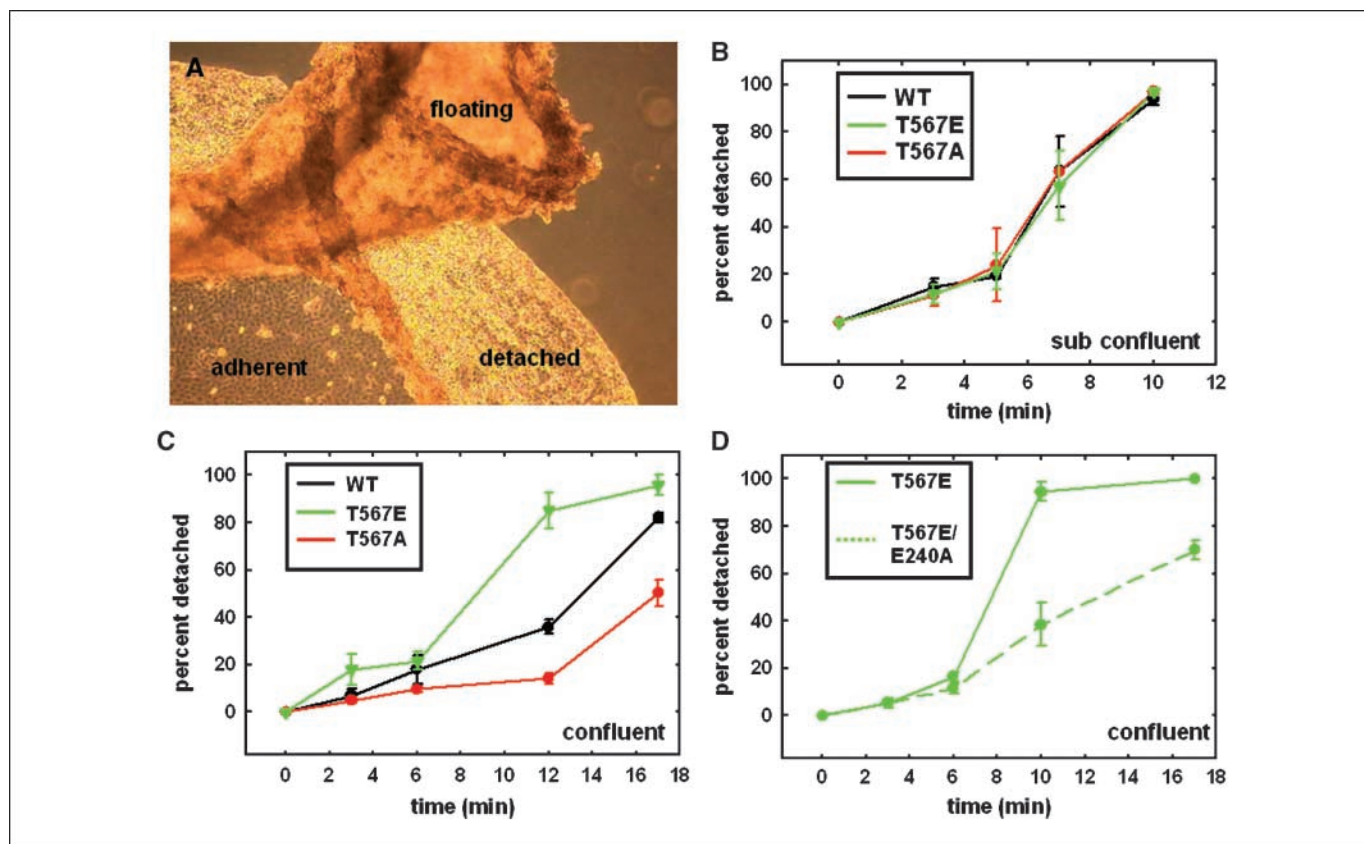
<sup>6</sup> M.V. Barbolina and M.S. Stack, unpublished results.

assay to monitor the kinetics of cell-matrix dissociation. In subconfluent cultures, detachment kinetics were similar in cells expressing wild-type, T567A-MT1-MMP, or T567E-MT1-MMP (Fig. 2B). On confluence, cells expressing T567E-MT1-MMP exhibit a more complete and accelerated detachment relative to cells expressing wild-type or T567A-MT1-MMP (Fig. 2C). Cells lacking MT1-MMP expression (vector controls) are strongly adherent and detach with extended kinetics (>20 min) under these conditions (data not shown). DOV13 cells do not exhibit spontaneous detachment; however, it should be noted that these cells also express high levels of the endogenous MMP inhibitor TIMP-2 (13, 15). To determine whether MT1-MMP catalytic activity was required for aggregate detachment, the active site mutation E240A was introduced into the T567E background to generate T567E/E240A-MT1-MMP-expressing cells. Detachment kinetics of T567E/E240A-MT1-MMP cells were significantly attenuated relative to cells expressing the catalytically active phosphomimetic mutant (Fig. 2D). Together, these results suggest that cell-cell communication regulates cell-matrix adhesion via the activity of MT1-MMP.

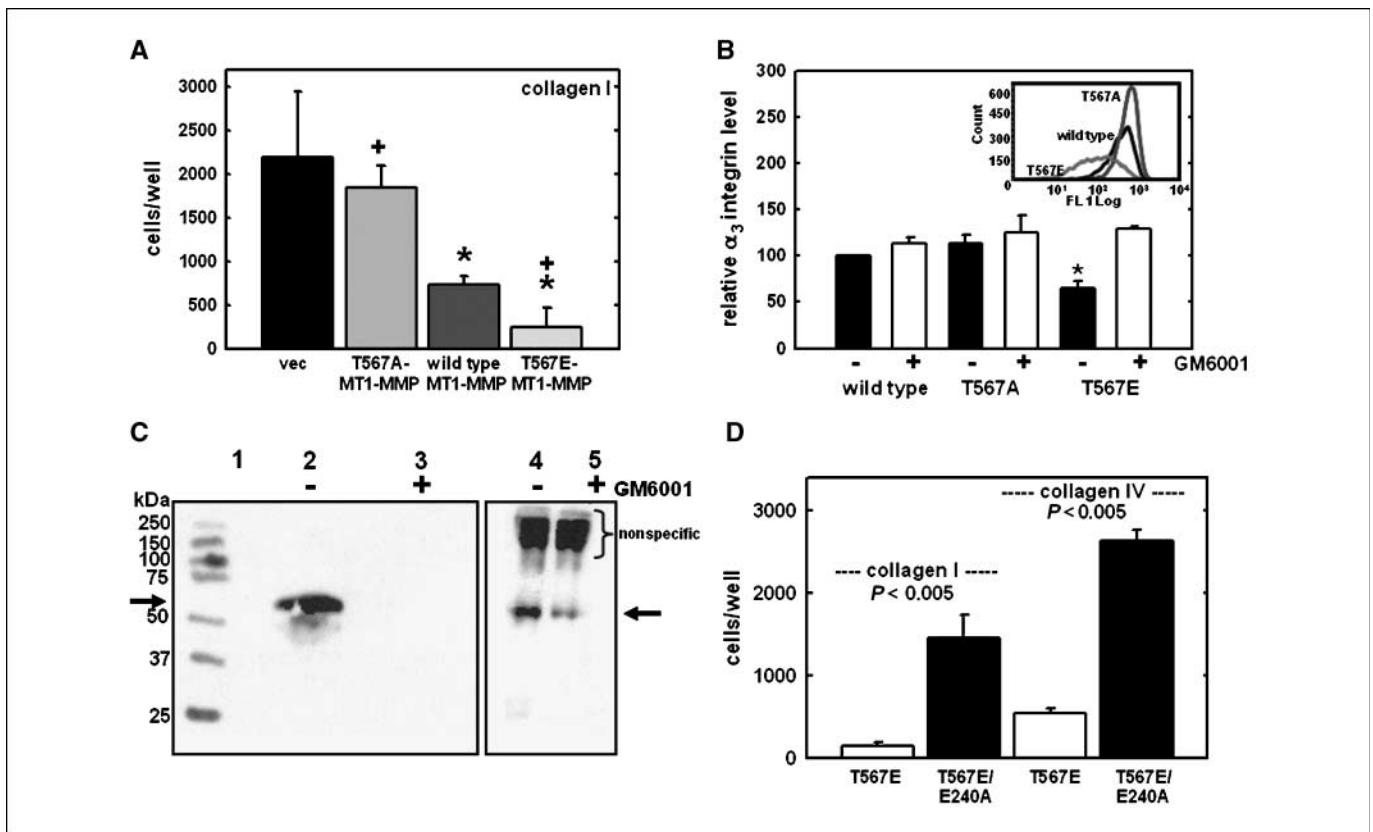
**MT1-MMP catalyzes  $\alpha_3$  integrin ectodomain shedding.** Reciprocal interplay between integrin and cadherin function has been reported (40, 41) and the results described above suggest that cadherin-engaged, confluent cells lose integrin function. Relative to phosphodeficient T567A cells, expression of both wild-type and T567E-MT1-MMP significantly impaired cell adhesion to both type I collagen (Fig. 3A) and type IV collagen (Supplementary Fig. S2A).

In contrast, adhesion to fibronectin was unaffected by MT1-MMP expression (Supplementary Fig. S2B), suggesting a specific defect in the expression and/or function of collagen-binding integrins. As detachment of cell-cell adherent sheets of T567E-MT1-MMP-expressing cells occurs on confluence (Fig. 2A), integrin profiles were compared under these conditions. A significant reduction in levels of cell surface  $\alpha_3$  integrin was observed in confluent cultures of T567E-MT1-MMP-expressing cells (Fig. 3B, inset, red trace) relative to cells expressing wild-type (Fig. 3B, inset, black trace) and T567A-MT1-MMP (Fig. 3B, inset, blue trace). Vector control cells maintain  $\alpha_3$  expression at levels seen in subconfluent cells (data not shown). To evaluate a potential role for MT1-MMP activity in this process, cells were cultured with GM6001 to block MT1-MMP catalytic activity. Inhibition of MMP catalytic activity rescues  $\alpha_3$  integrin surface expression in confluent cells expressing wild-type or T567E-MT1-MMP (Fig. 3B). In control experiments, surface levels of  $\alpha_3$  integrin were similar in subconfluent cells expressing wild-type, T567A-MT1-MMP, or T567E-MT1-MMP (Supplementary Fig. S3A). Additional controls show no loss of surface levels of  $\alpha_2$  integrin (Supplementary Fig. S3B),  $\beta_1$  integrin (Supplementary Fig. S3C), E-cadherin (Supplementary Fig. S3D), and transferrin receptor or integrins  $\alpha_v$ ,  $\alpha_5$ , and  $\alpha_6$  (data not shown) in subconfluent or confluent cells in the presence or absence of GM6001, suggesting that loss of  $\alpha_3$  integrin expression does not represent nonspecific loss of cell surface protein.

The ability of GM6001 to rescue surface expression of integrin  $\alpha_3$  indicates that the observed reduction in surface presentation may



**Figure 2.** MT1-MMP expression promotes detachment of cell-cell adherent monolayers and MCA formation. A, confluence initiates detachment of T567E-MT1-MMP-expressing cells. Image shows adherent and detached portions of confluent monolayer with floating aggregate. B-D, quantitation of detachment using controlled trypsinization. Kinetics of cell detachment were evaluated by using (B) subconfluent or (C, D) confluent cultures of cells. Experiments were performed in triplicate.



**Figure 3.** MT1-MMP expression modulates adhesion and  $\alpha_3$  integrin ectodomain shedding. *A*, quantitation of adhesion to type I collagen. Results represent averages of three independent experiments. \*,  $P < 0.005$ , relative to vector controls; +,  $P < 0.005$ , relative to cells expressing wild type MT1-MMP. *B*, the effect of MMP inhibition on  $\alpha_3$  integrin surface expression. OVCA433 cells were cultured +/- GM6001 (25  $\mu\text{mol/L}$ ) overnight prior to harvesting for FACS analysis of integrin  $\alpha_3$ . *Inset*, surface expression of integrin  $\alpha_3$  under confluent conditions was assessed by FACS analysis. *Blue trace*, T567A-MT1-MMP cells; *black trace*, wild type MT1-MMP cells; *red trace*, T567E-MT1-MMP cells. *C*, confluent OVCA433 cells expressing T567E-MT1-MMP were cultured in serum-free medium in the absence (*lanes 2 and 4*) or presence (*lanes 3 and 5*) of GM6001 (25  $\mu\text{mol/L}$ ) overnight. *Lanes 2 and 3*, conditioned media were concentrated and probed with mouse anti- $\alpha_3$  antibody. *Lanes 4 and 5*, immunoprecipitation of unconcentrated conditioned medium with mouse anti-integrin  $\alpha_3$  followed by western blotting for integrin  $\alpha_3$  extracellular domain. *Lane 1*, molecular weight markers in kDa 250, 150, 100, 75, 50, 37, 25. *Arrow*, migration position of the 70.8 kDa  $\alpha_3$  integrin immunoreactive band. *D*, catalytically inactive E240A/T567E-MT1-MMP mutation restores cell adhesion to type I and type IV collagen. Results are averages of three independent experiments.

be the result of a MT1-MMP-catalyzed cleavage event. To explore this possibility, conditioned medium from confluent T567E-MT1-MMP-expressing cells was concentrated and analyzed for the presence of polypeptides that cross-react with anti- $\alpha_3$  integrin antibodies. Western blot analysis of concentrated conditioned medium identified a protein of ~70.8 kDa and this species was undetectable in the medium of cells cultured overnight with GM6001 (Fig. 3C, compare *lanes 2 and 3*). Similar results were obtained following immunoprecipitation of nonconcentrated conditioned medium with anti- $\alpha_3$  integrin antibodies followed by Western blotting for  $\alpha_3$  integrin (Fig. 3C, *lanes 4 and 5*). Impaired collagen adhesion, spontaneous detachment, decreased surface  $\alpha_3$  integrin levels, and  $\alpha_3$  integrin ectodomain shedding were not observed in cells expressing the catalytically inactive T567E/E240A-MT1-MMP (Fig. 3D; data not shown). Together, these data support a mechanism for loss of adhesion subsequent to MT1-MMP-catalyzed  $\alpha_3$  integrin ectodomain shedding, particularly evident in phosphomimetic T567E-MT1-MMP-expressing cells.

**MT1-MMP enables matrix-embedded proliferative growth.** Successful *i.p.* metastasis requires proliferation within the confines of the interstitial collagen-rich submesothelial matrix to establish collagen-anchored *i.p.* secondary lesions (11, 42, 43). MT1-MMP is

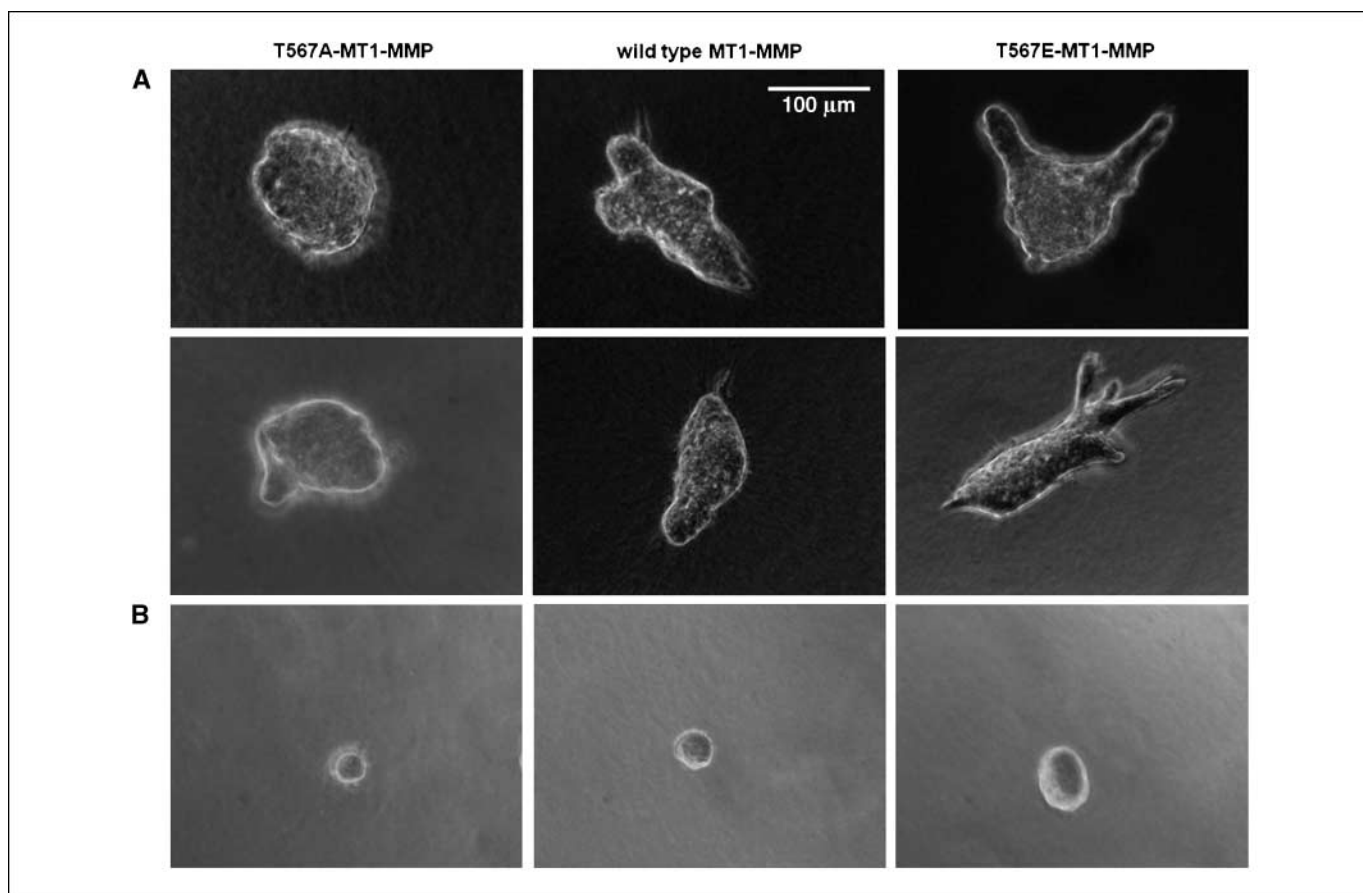
necessary for cell proliferation in a three-dimensional collagen network, functioning to remove cytoskeletal constraints necessary to drive a proliferative response (23). To examine the contribution of MT1-MMP activity to growth of ovarian cancer cells in three-dimensional collagen, cells were seeded at single-cell density in three-dimensional collagen gels and qualitatively assessed for growth after 6 days (31). Large MCA structures were generated by all MT1-MMP-expressing cells (Fig. 4A; Supplementary Table S1). Proliferation was significantly attenuated in vector-transfected control cells (data not shown) or by copolymerization of TIMP-2 within collagen gels (Fig. 4B), verifying the dependence of growth in three-dimensional collagen on MT1-MMP activity. DOV13 cells were also highly proliferative within three-dimensional collagen gels, forming clusters of cells averaging  $2.43 \pm 0.65 \times 10^5$  per cluster. Although overall structures generated by all MT1-MMP-expressing cells were similar in size (Supplementary Table S1), MCAs generated by T567A-MT1-MMP-expressing cells were more spherical (length/width ratio of 1.47) and lacked distinct foci of collagen invasion (Fig. 4A, *left*; Supplementary Table S1). Cells expressing wild-type MT1-MMP produced more elongated structures with small invasive foci (Fig. 4A, *middle*; Supplementary Table S1). In contrast, cells expressing the phosphomimetic mutant

T567E-MT1-MMP grew as multicellular prolate ellipsoid aggregates (length/width ratio of 2.16) with multiple large invasive foci (Fig. 4A, right; Supplementary Table S1). Quantitative analysis of invasive projections indicates that both projection number and projection length were significantly increased relative to cells expressing wild-type MT1-MMP (Supplementary Table S1). These results support the hypothesis that the phosphorylation status of MT1-MMP cytoplasmic residue T<sup>567</sup> may regulate invasive growth within the collagen-rich microenvironment of the submesothelial matrix. Furthermore, the data indicate that the ability of ovarian cancer cells to survive long-term and proliferate in three-dimensional collagen is enhanced by MT1-MMP activity, as proliferation is blocked by inclusion of TIMP-2 within the three-dimensional collagen gels (Fig. 4B). This is supported by an immunohistochemical analysis of MT1-MMP expression in sets of paired primary ovarian tumors and peritoneal metastatic lesions derived from the same patient ( $n = 17$ ; Fig. 5A–C; Supplementary Table S2). Although the data set is not sufficiently powered for rigorous statistical analysis, the data support a trend wherein 76% (13 of 17) show sustained or increased high-level (2+ and 3+) MT1-MMP expression in metastases. A further 12% (2 of 17) maintain low level (1+) expression in metastases, whereas only 12% (2 of 17) show decreased expression relative to the primary tumor. It is interesting to note that MT1-MMP is expressed in 100% of the metastatic lesions overall (Supplementary Table S2).

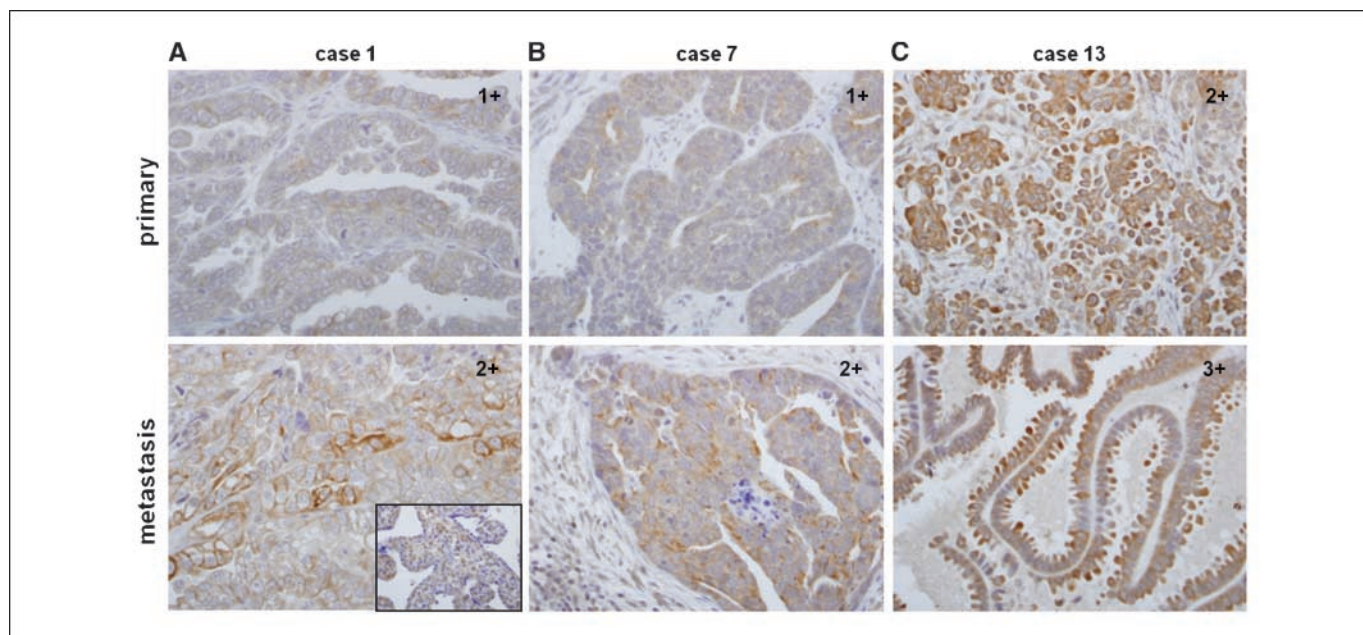
## Discussion

Reversible phosphorylation is widely recognized as a key post-translational modification that regulates protein function and a growing body of work suggests that MT1-MMP action may be altered through phosphorylation of cytoplasmic tail residues (26–28). For example, src-dependent or epidermal growth factor-dependent phosphorylation of cytoplasmic residue Y<sup>573</sup> has been shown to modulate cell migration and invasion (26, 28), suggesting that the intracellular domain of MT1-MMP may be vital to “inside-out” signaling processes. The observation that several cell surface proteins undergo phosphorylation at multiple sites underscores our observations implicating T<sup>567</sup> as a second site for post-translational modification (27). Although regulatory mechanisms for the control of T567 phosphorylation have yet to be elucidated, results in the present study highlight a pivotal role for the cytoplasmic tail in regulating MT1-MMP function and associated cellular phenotypes relevant to metastasis of ovarian carcinoma.

The majority of women with ovarian cancer are initially diagnosed with disseminated intra-abdominal disease (2, 3), indicating that a more detailed understanding of the cellular and biological factors that promote successful metastatic dissemination can ultimately improve patient survival. Unlike other solid tumors, hematogenous dissemination of ovarian cancer cells is uncommon, as metastasis proceeds primarily through exfoliation of cells from the primary tumor into the peritoneal cavity as free-floating cells



**Figure 4.** MT1-MMP promotes matrix-embedded proliferative growth. *A*, cells were seeded at an initial density of  $5 \times 10^4$  cells/well within 3D collagen gels and photographed after incubation for 6 d. *B*, in control experiments, TIMP-2 was co-polymerized within the gel at a final concentration of 5  $\mu$ g/mL.



**Figure 5.** Immunohistochemical analysis of MT1-MMP expression in paired primary ovarian tumors and peritoneal metastatic lesions. A–C, examples of paired primary tumor (*top*) and corresponding metastatic lesion (*bottom*) from the same patient stained with MT1-MMP-specific antibodies (1:100 dilution) and peroxidase-conjugated secondary antibody. *Inset*, positive control breast carcinoma tissue. *Numbers in top right corners*, immunohistological score of each tumor.

and MCAs. This imposes challenges to tumor cell survival because these metastatic cells must escape anoikis, and it has been speculated that MCA formation may function to promote anchorage-independent growth (5). Although originally considered a nonadhesive subset of ovarian tumor cells, recent data show that human ovarian cancer ascites-derived MCAs adhere to and invade mesothelial monolayers and can thereby contribute to i.p. implantation and metastasis (6, 7, 12). Further, MCAs that survive in ascites may generate a subpopulation of highly neoplastic cells (38). This is supported by recent data showing that cells isolated from murine ascites are more aggressive than parental cells when re injected *in vivo* (8). Gene expression profiling shows clustering of MCA expression profiles with tumor xenograft patterns, rather than with monolayer cells (9, 44), suggesting that MCAs represent a more advanced stage of malignancy. Self-renewing spheroid-forming cells isolated from ovarian primary tumors show increased tumorigenicity and are associated with metastasis to omentum and colon (10). Together, these data support the hypothesis that the MCA population in human ovarian ascites may be a primary source of i.p. metastases and thereby represents a key target for antimetastatic therapy.

Although recent studies highlight the importance of MCAs in ovarian cancer pathobiology, the process of self-assembly of ovarian tumor cells into microtissues such as MCAs has not been extensively evaluated. Further, it is unknown whether the temporal sequence of events in MCA formation involves shedding of individual tumor cells, which then aggregate in ascites versus exfoliation of multicellular tumor cell sheets that subsequently reorganize into MCAs. The current data suggest that acquisition of MT1-MMP activity would promote MCA formation by either mechanism. Cells expressing endogenous MT1-MMP (DOV13) readily aggregate from single cells into MCAs, and quantitative PCR and Western blotting analyses confirm elevated expression of MT1-MMP in MCA cultures. Alternatively, OVCA433 cells trans-

ferred with wild-type or T567E-MT1-MMP exhibit rapid detachment kinetics and sheet-like exfoliation as cell-cell adherent aggregates. Interestingly, this striking detachment phenotype is not manifested in subconfluent cultures, suggesting the potential contribution of cell-cell adhesion molecules to the regulation of cell-matrix adhesion. It is interesting to speculate that acquisition of MT1-MMP expression by primary tumor cells may promote metastasis via enhanced tumor cell shedding. In support of this hypothesis, previous immunohistochemical and *in situ* hybridization analyses show MT1-MMP expression in 78% to 100% of primary ovarian tumors (15, 18). MT1-MMP expression is also detected in cancer cells obtained from malignant effusions (17) and in 88% to 98% of peritoneal metastases, wherein expression is correlated with poor outcome (current study; ref. 17).

Detachment of cell-cell adherent sheets bears similarities to cohort migration (45). The ability of protease inhibitors to impede this process reinforces the role for MMP activity in establishing this phenotype. This is further supported by results from the current study showing that the catalytically inactive mutant T567E/E240A-MT1-MMP did not induce cell detachment and sheet-like exfoliation. Our data suggest that MT1-MMP catalytic activity contributes mechanistically to cell detachment via catalysis of  $\alpha_3$  integrin ectodomain shedding, as cell adhesion was restored and soluble integrin ectodomain was not detectable in the presence of a broad-spectrum MMP inhibitor or with the catalytically inactive mutant. It is plausible that shedding of cells as multicellular masses may represent a more efficient means of dissemination. In addition, the ability of cells to function collectively may allow the mass to produce high levels of matrix proteases that promote migration and invasion at secondary sites (45).

Metastasizing ovarian cancer cells encounter an interstitial collagen-rich microenvironment, as the submesothelial matrix is composed primarily of types I and III collagen (12, 46–50). Acquisition of MT1-MMP collagenolytic activity may be key to

metastatic success, as MT1-MMP activity is required for invasion of three-dimensional collagen gels by ovarian cancer cells (19). Furthermore, MT1-MMP collagenolysis is necessary to remove matrix barriers to allow for the cytoskeletal reorganization necessary to drive cellular proliferation (23). This is supported by results of the current study showing lack of proliferation within three-dimensional collagen gels in the absence of MT1-MMP expression or in gels containing TIMP-2. Although all MT1-MMP-expressing cultures formed proliferative colonies in three-dimensional collagen, cells expressing the phosphomimetic T567E-MT1-MMP construct exhibited more invasive patterns of growth. Thus, it is interesting to speculate that both the presence and the phosphorylation status of MT1-MMP may control metastatic success. Metastatic disease is the main cause of death for women with epithelial ovarian carcinoma, as disseminated tumor cells attach to abdominal surfaces, anchor, and grow multiple secondary lesions that disrupt the function of essential organs (1, 2). A molecular-level understanding of metastasis is necessary for the development of therapies to inhibit i.p. spread and thereby improve the long-term survival of thousands of women

with ovarian cancer. To this end, a more detailed understanding of what regulates the transition from primary tumor to free-floating MCA to life-threatening peritoneally anchored metastatic lesion may provide novel insight necessary to target i.p. therapies to appropriate multicellular populations.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

Received 10/28/08; revised 5/22/09; accepted 6/15/09; published OnlineFirst 8/25/09.

**Grant support:** NIH/National Cancer Institute grants CA86984 (M.S. Stack), CA86984-S1 (N.M. Moss), and CA109545 (M.S. Stack) and Illinois Department of Public Health Penny Severns Breast, Cervical and Ovarian Cancer Research Fund (M.V. Barbolina).

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We thank Dr. Brian Adley (Advocate Lutheran General Hospital) for scoring of immunohistochemical stains.

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