

c-Met Overexpression Is a Prognostic Factor in Ovarian Cancer and an Effective Target for Inhibition of Peritoneal Dissemination and Invasion

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Abstract

The hepatocyte growth factor receptor c-Met is a receptor tyrosine kinase that plays an important role in tumor growth by activating mitogenic signaling pathways. The goal of this study was to evaluate the role of c-Met in the biology of ovarian cancer and to determine its potential as a therapeutic target. c-Met protein expression was detected by immunohistochemistry in 138 advanced-stage ovarian cancers using a tissue microarray annotated with disease-specific patient follow-up. Fifteen of 138 (11%) tissues had c-Met overexpression. Median survival for patients with high c-Met levels was 17 months versus 32 months ($P = 0.001$) for patients with low c-Met expression. Infection of SKOV-3ip1 cells with an adenovirus expressing a small interfering RNA (siRNA) against c-Met efficiently inhibited c-Met protein and mRNA expression as well as extracellular signal-regulated kinase and phosphatidylinositol 3-kinase signaling. It also inhibited adhesion to different extracellular matrix components, human primary mesothelial cells, and full-thickness human peritoneum and, *in vivo*, to mouse peritoneum. This was paralleled by a significant reduction in α_5 and β_1 integrin protein and mRNA expression as well as a reduction of urokinase and matrix metalloproteinase (MMP)-2/MMP-9 activity. In SKOV-3ip1 ovarian cancer xenografts, *i.p.* treatment with the c-Met siRNA significantly reduced tumor burden, ascites formation, protease activity, and the number of peritoneal implants but not tumor size or angiogenesis. These results suggest that c-Met overexpression is a prognostic factor in ovarian cancer and that targeting c-Met *in vivo* inhibits peritoneal dissemination and invasion through an $\alpha_5\beta_1$ integrin-dependent mechanism. Therefore, c-Met should be explored further as a therapeutic target in ovarian cancer. [Cancer Res 2007;67(4):1670–9]

Introduction

Ovarian cancer has the highest mortality rate of all gynecologic tumors and is the fifth leading cause of cancer death among U.S. women. It is often diagnosed at a late stage after tumor cells are disseminated within the peritoneal cavity. Despite aggressive

treatment, such as surgical cytoreduction and chemotherapy, more than two thirds of all patients succumb to the disease within 5 years (1). A current working model for the metastatic process of ovarian carcinoma suggests that cancer cells are shed from the ovarian tumor into the peritoneal cavity and attach to the layer of mesothelial cells that line the inner surface of the peritoneum. The cancer cells invade the superficial layers of abdominal organs and may then spread to retroperitoneal lymph nodes and the pleural cavity (2, 3).

c-Met plays an important role in tumorigenesis. On binding of its ligand, hepatocyte growth factor (HGF)/scatter factor (SF), the receptor undergoes dimerization and autophosphorylation at specific tyrosine residues within the cytoplasmic domain, creating docking sites for intracellular signal transducers that activate the ras-mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K), and the signal transducers and activators of transcription (STAT) signaling pathway (4). The expression of c-Met has been studied in several epithelial and mesenchymal cancers, and in general, high expression of c-Met protein is an independent prognostic factor associated with an adverse outcome.⁸ We and others have shown that c-Met overexpression in breast tumors is associated with breast cancer progression and that high c-Met expression correlates with poor survival (5–7).

Several lines of evidence suggest that c-Met plays an important role in the transformation of surface ovarian epithelial cells and in the growth and dissemination of ovarian cancer. First, as ovarian epithelial cells acquire genetic changes at different stages of neoplastic progression, they express higher levels of c-Met (8, 9). Second, HGF/SF is highly expressed in ovarian cancer ascites inducing proliferation of cultured ovarian cancer cells (10). Third, a fragment of the HGF molecule, NK4, which functions as an inhibitor of c-Met/HGF signaling, abrogates ovarian cancer cell migration *in vitro* and delays *i.p.* tumor growth and peritoneal dissemination *in vivo* (11). Fourth, the expression of c-Met persists in cultured ovarian surface epithelial cells and its expression level is increased in ovarian cancer (12).

These results indicate that the c-Met/HGF system may be implicated in the biology of ovarian cancer and that it could be a promising target. However, previous studies with ovarian cancer cell lines focused on the role of c-Met in migration, cell viability, and protease overexpression. It is still not known whether c-Met

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⁸ <http://www.vai.org/vari/metandcancer/>

affects the peritoneal adhesion and dissemination of ovarian cancer cells, although these are key mechanisms of ovarian cancer metastasis. Moreover, the expression pattern of c-Met in ovarian cancer has only been characterized in small cohorts of patients who lacked clinical follow-up. With this in mind, we undertook a study with two objectives: (a) to determine the prognostic value of c-Met in a cohort of patients with advanced ovarian cancer and (b) to determine if silencing c-Met in ovarian cancer cell lines affects cell signaling, proliferation, peritoneal adhesion, invasion, and *in vivo* tumor formation.

We show that high expression of c-Met is associated with an adverse prognosis and that blocking c-Met expression by small interfering RNA (siRNA) inhibits adhesion, invasion, peritoneal dissemination, and tumor growth through an $\alpha_5\beta_1$ integrin-dependent mechanism.

Materials and Methods

Reagents and cell lines. CD44 monoclonal antibody (515), integrin α_5 , collagen type 1, fibronectin, and vitronectin were purchased from BD Biosciences (Bedford, MA). The c-Met antibody (3D4) was from Zymed (San Francisco, CA) and the phosphorylated c-Met (Tyr¹²³⁰/Tyr¹²³⁴/Tyr¹²³⁵) antibody was from BioSource (Camarillo, CA). c-Met (C-28), vascular endothelial growth factor (VEGF; A-20), β_1 integrin (P5D2), and α_2 integrin antibody (PIH5) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β_3 (B3A), β_4 (3E1), $\alpha_5\beta_1$ (JBS5), $\alpha_v\beta_3$ (LM609), and $\alpha_v\beta_5$ integrin (P1F6) antibodies were obtained from Chemicon (Temecula, CA). Antibodies against p44/42 MAPK, phosphorylated p44/42 MAPK, AKT, and phosphorylated AKT (Ser⁴⁷³) were from Cell Signaling (Beverly, MA). The Ki-67 (Sp6) antibody was from Lab Vision (Fremont, CA). The human ovarian cancer cell lines CAOV-3, SKOV-3, OVCAR-5, and HEK293 were from the American Type Culture Collection (Rockville, MD). OVMZ-6 cells were provided by Dr. Volker Möbus (Hospital Frankfurt-Höchst, Frankfurt, Germany), SKOV-3ip1 cells were from Dr. Gordon Mills (M. D. Anderson Cancer Center, Houston, TX), and A2780 cells were provided by Dr. Poruchynsky (National Cancer Institute, Bethesda, MD). The IOSE-T and IOSE-29 immortalized ovarian surface epithelial (IOSE) cell lines were provided by Dr. Nelly Auersperg (University of British Columbia, Vancouver, British Columbia, Canada).

Patients and tissue microarray. Tissue blocks from 161 patients with Federation Internationale des Gynaecologistes et Obstetristes (FIGO) stage III/IV advanced ovarian cancer and 27 patients with stage I/II early ovarian cancer who had undergone surgery by a gynecologic oncologist at the University of Chicago (Chicago, IL) between 1994 and 2004 were selected for the study after Institutional Review Board approval was obtained. Samples of tissue sections were stained with H&E, and 1.5-mm cores were punched from donor blocks, inserted into a recipient block, and stained with H&E to confirm the presence of tumor. Satisfactory immunohistochemical staining of c-Met was obtained in 138 patients.

Immunohistochemistry. Tissue microarray slides were deparaffinized in xylene and hydrated with alcohol before being placed in 3% H₂O₂/methanol blocking solution, which was followed by antigen unmasking. Incubation with the antibody against total c-Met was done with a 1:100 dilution. The slides were stained using the EnVision avidin-biotin-free detection system and counterstained with hematoxylin. Immunoscoring was done using the Cellular Image Analysis System (ACIS, Clariant, San Juan Capistrano, CA). Within the tissue core, the most representative tumor area of standardized size was selected at $\times 100$ magnification. The staining intensity in this area was measured if the area contained $>5\%$ epithelial cells. Stains were scored as negative (–), weak (+), intermediate (++), and strongly positive (+++) as reported (5, 6, 13).

c-Met siRNA adenovirus infection. The c-Met siRNA expression plasmids and the replication-deficient c-Met siRNA adenoviral vectors (si-hMet-Ad5²²¹) have been recently described (14). Adeno-X-LacZ adenovirus was used as a control vector and as a marker of infection efficiency.

Western blot analysis. Cells were starved before being stimulated with HGF/SF (40 ng/mL). Tumor tissues from mice experiments were snap frozen, grinded, and lysed with ice-cold radioimmunoprecipitation assay buffer. An equal amount (15 μ g) of cell extracts was separated by SDS-PAGE. Proteins were visualized with enhanced chemiluminescence.

Reverse transcription-PCR analysis. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA). One-step reverse transcription and PCR were done using the SuperScript III and Platinum Taq DNA Polymerase (Invitrogen). RNA (1 μ g) was used for each reaction. The primers used were as follows: Met, 5'-AGCCAGTAATGATCTCAATAG-3' (sense) and 5'-TCAGGATAGGGGACAGGT-3' (antisense); β_1 integrin, 5'-CAACCGTAGCAAAGGAACAGCA-3' (sense) and 5'-AAATCCAATTCTG-AAGTCCG-3' (antisense); α_5 integrin, 5'-GCTCGAGAGGATTGCAGAGA-3' (sense) and 5'-GAGGGACTGTAACCGAAGG-3' (antisense); and actin, 5'-CGTGACATCAAAGAGAAGCTGTG-3' (sense) and 5'-GCTCAGGAGGAG-CAATGATCTTGA-3' (antisense). The cDNA synthesis was done at 55°C for 30 min. The PCR conditions were as follows: 94°C for 2 min followed by 25 cycles at 94°C for 15 min, 55°C for 30 s, and 68°C for 30 s. The final extension occurred at 68°C for 5 min.

Flow cytometry. Cells suspended in DMEM with 10% fetal bovine serum were incubated with the primary antibodies (1:250) for 30 min at 4°C followed by phycoerythrin (PE)-labeled secondary antibody. After washing, cells were resuspended in 500 μ L of 1% bovine serum albumin (BSA)/PBS. Isotypic mouse IgG was used as a negative control. Surface expressions of integrins and CD44 were measured with a FACSCalibur (Becton Dickinson, San Jose, CA).

Primary culture of human peritoneal mesothelial cells. Specimens of human peritoneum were obtained from patients undergoing surgery for benign conditions and mesothelial cells were extracted as described (15). To obtain a monolayer of human peritoneal mesothelial cells (HPMC) on a 96-well plate, 1.5×10^4 cells were cultured until they became confluent.

***In vitro* adhesion assay to ECM components and human peritoneum.** Adenovirus-infected SKOV-3ip1 cells were fluorescently labeled with either 10 μ Mol/L CMTPX (red) or CMFDA (green; Molecular Probes, Eugene, OR). Cells (5×10^4) were plated in a 96-well plate precoated with collagen type 1 (50 μ g/mL), fibronectin (5 μ g/mL), vitronectin (5 μ g/mL), or a monolayer of HPMCs. When a blocking antibody was used, cells were starved with 10 μ g/mL of the antibody (JBS5) or the equivalent control mouse IgG overnight. Cells were then detached from the plate using a nonenzymatic cell dissociation buffer, labeled with CMTPX, and plated. After incubation for 1 h at 37°C, cells were washed and fixed. The number of adhesive cells was quantified by measuring the fluorescent intensity (excitation, 590 nm; emission, 620 nm) with a fluorescence spectrophotometer. Full-thickness human peritoneum was excised during surgery and placed in a 24-well plate filled with 1.0 mL of 0.1% BSA/DMEM. Fluorescently labeled SKOV-3ip1 cells were overlaid on peritoneum and the plate was incubated at 37°C for 60 min. After washing, the cells adherent to the peritoneum were lysed with 1% NP40 and fluorescence intensity was measured.

***In vivo* cell adhesion to peritoneum.** Adenovirus-infected SKOV-3ip1 cells were fluorescently labeled and 2×10^6 cells were injected into the peritoneal cavity of female athymic nude mice. After 4 h, mice were sacrificed and the full peritoneum was excised and placed in a 24-well culture plate. Adherent cells were lysed with 1% NP40 and fluorescence was measured.

Matrigel invasion assay and zymograms. *In vitro* invasion was assayed by determining the ability of cells to invade through Matrigel (16). Zymograms were done as previously reported by us (17, 18).

Animals. SKOV-3ip1 cells (1.0×10^6) were injected i.p. into female athymic nude mice, and 1 week after injection, the si-hMet-Ad5²²¹ [1.0×10^9 plaque-forming units (pfu)/mouse], the control adenovirus, or an equivalent volume of PBS was injected twice weekly for 4 weeks of treatment (10 mice per group). After sacrifice, ascites was quantified, the number of colonies was counted, all visible colonies were dissected, and the removed tumor was weighed.

Statistical analysis. Tests for association between c-Met and clinicopathologic variables were computed using the Mantel-Haenzel χ^2 test or Fisher's exact test. Survival estimates were computed using the Kaplan-Meier

method, and comparisons between groups were analyzed using the log-rank test. Univariate analysis and multivariable models were fit using a Cox proportional hazards regression model. A forward inclusion approach was used to fit the multivariable model so that variables achieving significance at the $\alpha = 0.05$ level would be included. Wilcoxon signed rank tests were used to test for c-Met expression differences between primary tumors and metastases.

Results

c-Met overexpression as a prognostic marker in ovarian cancer. c-Met protein expression was evaluated in 138 patients with advanced ovarian or peritoneal cancer (stage III/IV) by staining a tissue microarray with a c-Met antibody. The c-Met staining localized to both the cytoplasm and the cell membrane and most tumors showed a uniform staining pattern in the

epithelial component but not in the stroma (Fig. 1A). Tumors rarely showed nuclear staining, a finding that is also described for other receptor tyrosine kinases (19). In accordance with previous reports on c-Met staining (5, 13) and our own experience (6), overexpression was defined as strong staining (+++) of c-Met in the tumor. Fifteen (11%) patients met this stringent definition of c-Met overexpression, including 11 of 114 serous, 3 of 10 clear cell, and 1 of 3 mucinous cancers. None of the 11 endometrioid cancers expressed c-Met. To determine if c-Met overexpression occurs during early or late ovarian cancer progression, we stained tissue from 27 patients with early ovarian cancer (stage I/II). Four (15%) patients had c-Met overexpression, a percentage that is similar to advanced ovarian cancer, suggesting that Met expression occurs early in ovarian cancer development.

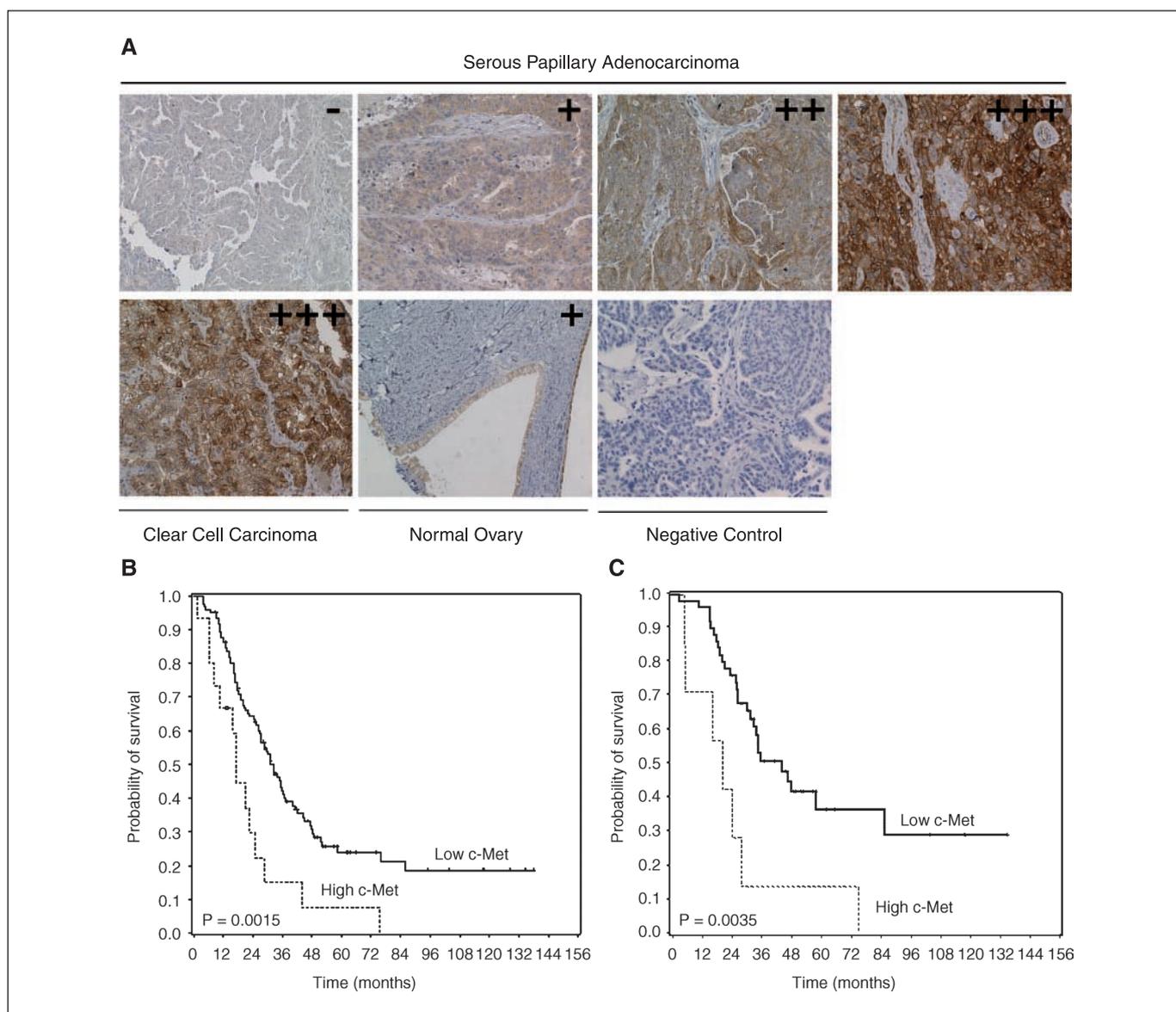


Figure 1. Immunohistochemical staining of a tissue microarray with malignant and normal ovarian tissue sections. **A**, representative areas of four different serous papillary ovarian cancers stained using a monoclonal anti-human c-Met antibody and scored as 0, 1, 2, and 3+. A strong positive staining is seen in a clear cell cancer, whereas ovarian surface epithelial cell staining in a normal ovary was weak. No positive signal was observed by nonimmune sera. Original magnification, $\times 250$. **B**, c-Met overexpression correlates with poor prognosis in patients with FIGO stage III/IV advanced ovarian cancer. Kaplan-Meier curves of overall survival in all ($n = 138$) patients. **C**, overall survival in optimally debulked patients with FIGO stage III/IV ovarian cancer who did not receive neoadjuvant therapy ($n = 58$).

To determine whether c-Met is a prognostic factor in ovarian cancer, univariate analysis was done and c-Met expression was compared with traditional ovarian cancer markers. Patients with high c-Met expression had a significantly poorer prognosis than those without c-Met overexpression ($P = 0.0015$). The median overall survival in women with strong c-Met expression was 17 months (10.2–24.6), whereas it was 32 months (26.9–36.7) in patients with low c-Met expression (Fig. 1B). There was no statistically significant difference in progression-free survival between patients whose tumors had high or low c-Met expression. Of the traditional markers of tumor aggressiveness, the size of the residual tumor left at the end of surgery and stage were significant predictors of overall and progression-free survival (Table 1) as has been previously reported (1). For the multivariate analysis, we used all clinicopathologic variables and c-Met expression to select a model for overall survival with multiple predictors. The final model indicates that residual disease and c-Met protein expression are independent prognostic indicators for overall survival ($P = 0.002$ and 0.005 , respectively).

Although optimal debulking is associated with longer survival, at least 60% of ovarian cancer patients whose tumor could be resected to a size of 1 cm or smaller develop recurrent disease. Because it is clinically important to predict which patients in this subgroup will suffer a recurrence, we sought to determine whether c-Met is associated with survival. Kaplan-Meier analysis showed that, despite optimal surgical debulking, patients with c-Met overexpression have a decreased progression-free survival ($P = 0.04$; data not shown) and overall survival ($P = 0.0035$; Fig. 1C). These findings suggest that c-Met is an independent factor in this subgroup of patients who are potentially curable. Several reports suggest that c-Met is involved in metastasis (6, 20), but this has not been studied in human ovarian cancer. When comparing c-Met expression between the primary ovarian tumor and the corresponding omental ($n = 75$; $P = 0.49$) or peritoneal ($n = 60$; $P = 0.51$) metastases, we found no significant difference in expression.

Inhibition of c-Met expression in ovarian cancer cell lines by siRNA. Because c-Met is a prognostic factor in ovarian cancer, we sought to determine whether c-Met inhibition is a feasible strategy for ovarian cancer treatment. c-Met is strongly expressed in six of seven ovarian cancer cell lines and weakly expressed in two IOSE cell lines (Fig. 2A). Only the A2780 ovarian cancer cell line did not show c-Met expression, which is consistent with a previous report (21). Four ovarian cancer cell lines (SKOV-3, SKOV-3ip1, CAOV-3, and OVCAR-5) that overexpress c-Met were infected with an adenovirus expressing a siRNA against human c-Met (si-hMet-Ad5; ref. 14). The siRNA efficiently inhibited c-Met protein and mRNA expression, whereas a control virus expressing enhanced green fluorescent protein had no effect (Fig. 2B and C). All cell clones showed similar expression levels of actin protein and mRNA.

Several studies have established that HGF/SF stimulation leads to the activation of c-Met by phosphorylation of the cytoplasmic domain and also activation of the MAPK, PI3K, and STAT signaling pathway(s), which mediate the multiple biological effects of c-Met (22–24). We therefore asked whether they are activated by HGF/SF and whether they can be inhibited by the c-Met siRNA (Fig. 2D). In the absence of HGF/SF, c-Met was not phosphorylated. Addition of HGF/SF induced tyrosine phosphorylation, an effect that was completely abrogated by infection of the cells with the c-Met siRNA. This effect was paralleled by the partial inhibition of basal extracellular signal-regulated kinase (ERK) 1 and complete inhibition of ERK2 phosphorylation, which was sufficient to

Table 1. Patient characteristics ($n = 138$)

		<i>P</i>
Median age, y (range)	59 (36–87)	
Median observation time of patients alive, mo (range)	41 (8–137)	
FIGO stage, <i>n</i> (%)		
III	94 (68.1)	0.015
IV	44 (31.9)	
Disease, <i>n</i> (%)		
Ovarian cancer	114 (82.6)	0.053
Primary peritoneal cancer	24 (17.4)	
Histologic subtype, <i>n</i> (%)		
Serous papillary adenocarcinoma	114 (82.6)	0.075
Endometrioid adenocarcinoma	11 (8.0)	
Clear cell carcinoma	10 (7.2)	
Mucinous carcinoma	3 (2.2)	
Histologic grade, <i>n</i> (%)		
G ₁ + G ₂	42 (30.4)	0.878
G ₃	96 (69.6)	
Residual tumor (cm), <i>n</i> (%)		
≤1	72 (52.2)	0.001
>1	62 (44.9)	
Unknown	4 (2.9)	
Chemotherapy, <i>n</i> (%)		
Taxane/platinum	115 (83.3)	0.002
Platinum only	3 (2.2)	
Other/platinum	13 (9.4)	
None	7 (5.1)	
Chemotherapy type, <i>n</i> (%)		
Neoadjuvant	22 (15.9)	0.338
Primary	109 (79.0)	
None	7 (5.1)	

NOTE: *P* values were calculated with the log-rank tests to determine if the clinicopathologic factors are predictors of overall survival.

prevent activation of ERK1/ERK2 by HGF/SF. To evaluate the activity of the PI3K signaling pathway, AKT phosphorylation was detected. The basal as well as the inducible activation of AKT were inhibited by the c-Met siRNA, whereas total AKT expression was not affected. The phosphorylation of p38MAPK, STAT3, and STAT5 in SKOV-3ip1 was constitutively high, probably reflecting the strong overexpression of HER-2/*neu* in this cell line (25). It was not further activated by HGF/SF nor inhibited by the c-Met siRNA (data not shown).

Inhibition of c-Met reduces adhesion of ovarian cancer cells to abdominal peritoneum through an integrin-mediated mechanism. Ovarian cancer metastasizes primarily on the peritoneum and rarely to distant sites. Subsequent peritoneal implants are characterized by the adhesion and invasion of tumor cells into the peritoneum, leading to miliary dissemination. Mesothelial cells cover the abdominal cavity and all internal organs and lie on an extracellular matrix (ECM) containing fibronectin, vitronectin, and collagen (3, 26, 27). The mesothelium is the first surface encountered by tumor cells, and successful adhesion to mesothelial cells is important in the metastasis formation of ovarian cancer cells (28). Given that c-Met mediates epithelial cell scattering and migration, we reasoned that its inhibition might affect ovarian cancer cell adhesion to the peritoneum.

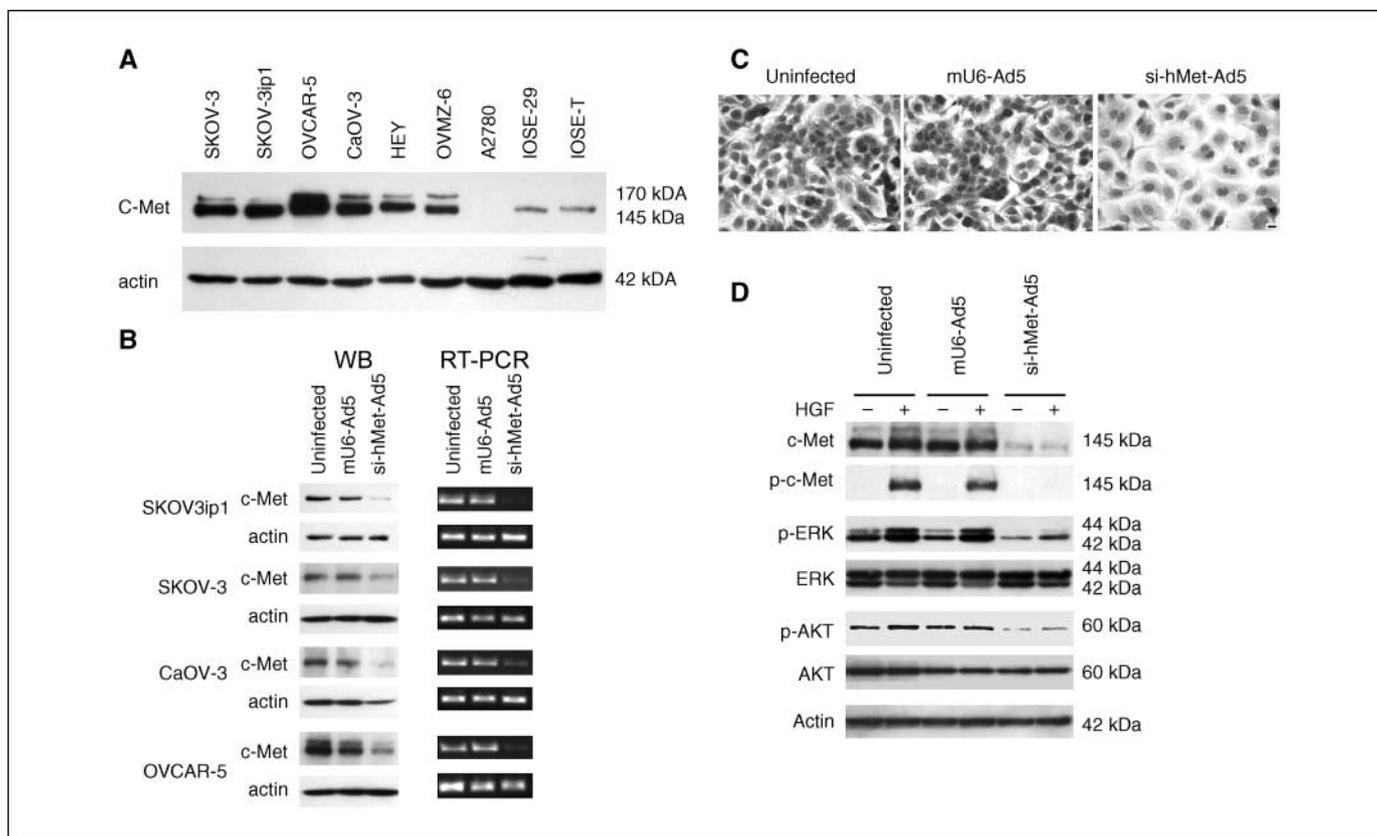


Figure 2. A c-Met siRNA inhibits c-Met expression and Met signaling in ovarian cancer cells. *A*, expression of c-Met in ovarian cancer cell lines and IOSE cells. *Top*, expression of c-Met was analyzed by immunoblotting using a human c-Met antibody. The band at ~170 kDa represents the single-chain precursor form of glycosylated c-Met. The band at ~145 kDa represents the β -chain of the biologically active mature c-Met heterodimer. *Bottom*, the membrane was stripped and reprobbed with β -actin as internal loading control. Blots are representative of three experiments. *B*, c-Met protein (*left*) and mRNA (*right*) expression is inhibited by a c-Met siRNA in four ovarian cancer cell lines. Human ovarian cancer cells were infected with control adenovirus (mU6-Ad5) or si-hMet-Ad5 at a multiplicity of infection of 100. After 3 d, cells were harvested and c-Met expression level was detected by immunoblotting and RT-PCR. *C*, detection of c-Met in SKOV-3ip1 cells by immunohistochemistry. Representative examples of uninfected, control adenovirus-infected (mU6-Ad5), and si-hMet-Ad5-infected (siRNA) SKOV-3ip1 cells. *Bar*, 20 μ m. *D*, effect of the c-Met inhibiting siRNA on Met phosphorylation and signaling. SKOV-3ip1 cells were infected with the si-hMet-Ad5 as described above and stimulated with HGF/SF (40 ng/mL) for 10 min. Cell lysates were collected and equal amount of cell lysates (15 μ g) was resolved by 10% SDS-PAGE and immunoblotted with anti-phosphorylated c-Met (*p-c-Met*) antibody, anti-phosphorylated p44/42 MAPK antibody, or anti-phosphorylated AKT (*p-AKT*) antibody. The membranes were stripped and rehybridized with antibodies detecting the total form of the protein or β -actin. Blots are representative of three experiments.

To determine the effect of the c-Met siRNA on adhesion, SKOV-3ip1 cells were infected with si-hMet-Ad5, labeled fluorescently, and then plated on different ECM components. Adhesion to fibronectin, vitronectin, and collagen I was significantly inhibited by the c-Met siRNA, whereas the control virus had no effect. Because the peritoneal ECM is covered by mesothelium, adhesion of ovarian cancer cells to primary human mesothelial cells was tested after infection with the c-Met siRNA. Adhesion of SKOV-3ip1 cells to mesothelial cells was abrogated by 43%, a significantly larger inhibition than the 20% to 40% inhibition found in adhesion to ECM molecules (Fig. 3A).

In view of these findings, we did two sets of experiments to determine if the siRNA can also reduce *in vivo* adhesion. First, we labeled both c-Met siRNA and control-infected cells with different fluorescent dyes and allowed them to adhere to human peritoneum removed from patients at surgery. Confirming and extending the *in vitro* results, the c-Met siRNA-infected cells were not able to adhere to the full-thickness peritoneum as efficiently as the control virus-infected cells (Fig. 3B). Subsequently, we determined whether the siRNA inhibits adhesion of SKOV-3ip1 cells after injection into the mouse peritoneal cavity. The c-Met siRNA-infected cells showed a 86% decrease in peritoneal binding,

whereas SKOV-3ip1 cells infected with the viral control attached to the mouse peritoneum efficiently.

The first step in ovarian cancer metastasis is adhesion, which is then followed by the invasion of ovarian cancer cells into the peritoneum. We therefore determined whether c-Met inhibition also affects invasion. A significant decrease in Matrigel invasion was observed in SKOV-3ip1 cells (Fig. 3C) and CAOV-3 cells (data not shown) infected with the c-Met siRNA-expressing adenovirus but not with the control virus. Infection with the c-Met adenovirus reduced urokinase activity in a casein/plasminogen-containing gel, whereas deletion of plasminogen abolished the band, indicating that the proteolytic activity could be ascribed to a plasminogen activator (Fig. 3D). This was paralleled by a reduction in urokinase and urokinase receptor mRNA as detected by reverse transcription-PCR (RT-PCR; data not shown). The inhibition of c-Met also inhibited the secretion of both pro-matrix metalloproteinase (MMP)-9 and active MMP-9 as well as pro-MMP-2 activity detected in a gelatin zymogram (Fig. 3D). These data suggest that c-Met is an important mediator of ovarian cancer cell adhesion and affects key proteases that are important for the invasion of ovarian cancer cells to the peritoneal cavity.

Adhesion of ovarian cancer cells to mesothelial cells is mediated by integrins and the hyaluronic acid receptor CD44 (3, 29, 30). Because the previous experiments suggested c-Met involvement in adhesion, we tested the possibility that the c-Met siRNA inhibits the expression of adhesion receptors. Although CD44 and several α and β integrins are expressed on SKOV-3ip1 cells, only β_1 and α_5 integrin expressions were significantly inhibited by the c-Met siRNA (Fig. 4A). The inhibition of β_1 and α_5 integrin expression in si-hMet-Ad5-infected cells was paralleled by a reduction in β_1 and α_5 integrin mRNA (Fig. 4B). Treatment of SKOV-3ip1 cells with an antibody against $\alpha_5\beta_1$ integrin reduced adhesion of SKOV-3ip1 cells to primary human mesothelial cells by 61% (Fig. 4C), giving further support to the hypothesis that c-Met mediates adhesion of ovarian cancer cells to the peritoneum by an $\alpha_5\beta_1$ integrin-dependent mechanism.

A c-Met siRNA inhibits tumor growth *in vivo*. Finally, we evaluated the efficacy of i.p. injection of the c-Met siRNA against SKOV-3ip1 cells growing in the peritoneal cavity of nude mice. Eight days after tumor cell inoculation, treatment with si-hMet-Ad5 was initiated twice weekly and continued for 4 weeks. The tumor distribution after 5 weeks resembled the clinical picture of human ovarian cancer with multiple tumors on the peritoneal surface, the omentum, the small bowel mesentery, and in both ovaries (Fig. 5A). The tumor burden was significantly higher ($P < 0.05$) in the PBS-injected and control virus-injected mice than in the siRNA-treated group (PBS, 1.02 ± 0.54 g; control, 1.01 ± 0.42 g; siRNA, 0.23 ± 0.22 g). The c-Met siRNA inhibited the formation of ascites, whereas the abdominal circumference of the mice treated with PBS or the control virus was notably increased by ascites (Fig. 5B). The ascites volume was significantly correlated with tumor weight, with a correlation coefficient of 0.72 ($P < 0.0001$). Inhibition of c-Met did not affect VEGF expression, a permeability and angiogenesis factor involved in ascites formation (31), nor did it affect vessel density as determined by CD31 staining (data not shown).

The number of intra-abdominal metastases was significantly lower in siRNA-treated mice (mean, 11.6 ± 19.8 lesions) when compared with control-treated mice (mean, 94.8 ± 48.8 lesions). However, although there were fewer metastases in the siRNA-treated mice, the tumor nodules that did grow had the same size and volume as those found in the control- or PBS-treated mice (Fig. 5A). This observation suggested that c-Met did not affect proliferation. Indeed, staining of i.p. tumors with the proliferation marker Ki-67 showed no difference between control groups and the siRNA-treated tumors (Fig. 5C). Because the Ki-67 staining reflects only the proliferation index at the end of the experiment, we sought to confirm this result *in vitro* and found that treatment of SKOV-3ip1 and CAOV-3 cells with the siRNA did not affect cell proliferation (Fig. 5C). Although proliferation was not affected by c-Met inhibition, the tumors showed a reduction in both urokinase and MMP-2/MMP-9-associated proteolytic activity (Fig. 5D), confirming our *in vitro* findings (Fig. 3D).

Taken together, these data are consistent with the hypothesis that c-Met affects peritoneal dissemination and invasion, but not tumor growth, and that, once the tumors attach successfully to the peritoneum, proliferation and angiogenesis are driven by Met-independent signaling pathways.

Discussion

The expression pattern and biology of c-Met have been studied in various tumors, but, as yet, these findings have not been

incorporated into the treatment of patients with cancer. Most of the tumor types analyzed (breast, lung, and colon) metastasize through hematogenous, lymphatic, or direct extension, whereas ovarian cancer metastasizes by peritoneal dissemination and, presumably, uses different molecular mechanisms (2). We undertook this study to characterize the expression pattern of c-Met in patients with ovarian cancer and to determine whether c-Met is a potential target in ovarian cancer treatment. We report here that c-Met overexpression is a prognostic marker for human ovarian cancer and that a siRNA against c-Met reduces tumor burden and peritoneal dissemination *in vivo*.

DiRenzo et al. (12) analyzed c-Met expression by Western blotting in fresh tissue from 67 patients with ovarian cancer and found that 21% had an intermediate expression of c-Met and 7% had a very strong expression. We found a similar expression pattern of c-Met using immunohistochemistry: of 138 patients, 27% had an intermediate and 11% had a strong expression of c-Met. In our study, strong c-Met expression correlated with lower overall survival. DiRenzo et al. (12) found no differences in the survival of patients stratified by c-Met expression; however, the small number of patients and short follow-up may have precluded finding a difference. We also found an interesting association between strong c-Met expression and histologic subtype. Most tumors with strong c-Met expression were found to have a serous papillary histology, but 3 of 10 clear cell cancers showed strong c-Met expression, suggesting that c-Met is highly expressed in this rare but clinically aggressive histologic subtype. This finding expands on those of previous studies that have reported the expression of c-Met in clear cell cancers (32) and renal cell cancer, which resembles clear cell cancer in its histologic appearance (33). Yet, although we believe that we have established c-Met overexpression as a marker for human ovarian cancer, we are aware that the percentage of patients with strong c-Met expression (11%) may seem too low to justify developing c-Met as a therapeutic target. However, it is probable that in the future ovarian cancers will be subclassified by means of such markers into smaller groups receiving targeted treatments as is currently done with HER-2/*neu* in breast cancer. Moreover, if the inhibition of c-Met shows a dose-dependent effect, subgroups of ovarian cancer patients with intermediate (27%) or weak c-Met expression (43%) may also benefit from the siRNA treatment.

Because ovarian cancer spreads by seeding tumor cells onto the peritoneum and because c-Met signaling increases cellular adhesion to ECM, we focused our investigation on the effect of c-Met inhibition on adhesion. Inhibition of c-Met reduced the binding of cancer cells to primary mesothelial cells and to fibronectin and collagen. Infection of SKOV-3ip1 cells with the c-Met siRNA was sufficient to inhibit adhesion to human peritoneum and *in vivo* adhesion to mouse peritoneum, highlighting the context dependency of c-Met function. This effect was paralleled by a reduction in α_5 and β_1 integrin expression, indicating that these integrins are regulated by c-Met. Treatment of SKOV-3ip1 cells with an $\alpha_5\beta_1$ integrin blocking antibody inhibited adhesion to a similar degree as treatment with the c-Met siRNA. A role for β_1 integrin in ovarian cancer cell adhesion is given further support by reports from other investigators: β_1 integrin-inhibiting antibodies reduced migration and adhesion as well as the binding of ovarian cancer cell lines to peritoneal mesothelial cells (29, 30, 34).

Several other integrins, and also CD44, were expressed in the SKOV-3ip1 cell line. However, they were not repressed by the

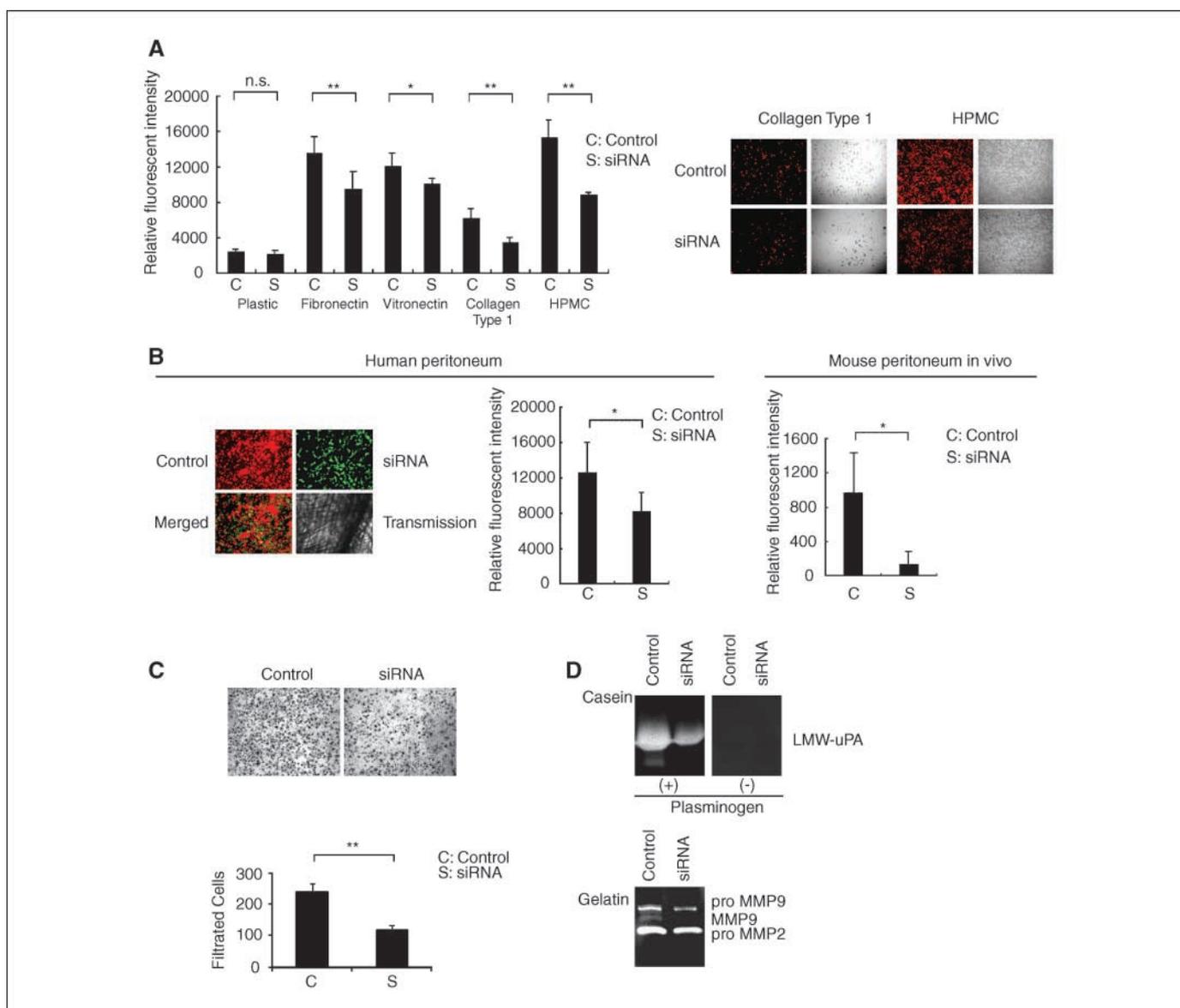


Figure 3. A siRNA inhibiting c-Met decreases *in vitro* and *in vivo* adhesion and invasion. **A**, adhesion of SKOV-3ip1 cells to ECM-coated wells. Control-infected and si-hMet-Ad5²²¹-infected cells were labeled with a fluorescent dye (CMTPX), resuspended in serum-free medium, and plated onto 96-well plates coated by passive absorption with fibronectin, vitronectin, collagen type 1, or a monolayer of primary HPMCs. After incubation for 1 h at 37°C, plates were washed to discard nonadherent cells and the number of adherent cells was quantified by measuring fluorescence intensity with a fluorescence spectrophotometer (excitation, 590 nm; emission, 620 nm). *Columns*, mean of three independent experiments. *Right*, representative image of labeled cells that attached to type I collagen or HPMC. **B**, *left*, si-hMet-Ad5-infected cells were fluorescently labeled with CMTPX (*green*) and control virus-infected cells were labeled with CMFDA (*red*). An equal number of cells (2.5×10^5 each) were incubated with excised human peritoneum at 37°C for 60 min. After the nonadherent cells were removed, adherent cells were observed under a fluorescent microscope. *Middle*, adhesion of SKOV-3ip1 cells to primary human peritoneum removed at surgery. Control adenovirus-infected and si-hMet-Ad5-infected cells were labeled with a fluorescent dye (CMTPX) and incubated at 37°C for 60 min on fresh full-thickness peritoneum removed at surgery. Adherent cells were lysed with 1% NP40, and the fluorescence intensity was measured as described above. The assays were carried out in triplicate. *Right*, effects of a c-Met RNA interference (RNAi) on peritoneal adhesion of SKOV-3ip1 cells *in vivo*. Adenovirus-infected SKOV-3ip1 cells were labeled with CMTPX and resuspended in PBS. Cells (2×10^6) were injected into the peritoneal cavity of nude mice, and after 4 h, mice were sacrificed and the peritoneum was dissected from the anterior abdominal wall. The peritoneum was gently washed in PBS to remove nonadherent cells, punch biopsies of equal diameter were placed in a 24-well culture plate and lysed with 1% NP40 for 4 h, and fluorescence was measured. The experiment was repeated thrice. *, $P < 0.05$; **, $P < 0.01$. **C**, *in vitro* invasion assays were done using a modified Boyden chamber system. Equal numbers (5×10^4) of infected cells were placed on the top chamber and allowed to invade for 24 h. Representative photographs are shown at low magnification. The results were from four independent experiments. **, $P < 0.01$. **D**, c-Met siRNA inhibits proteolytic activity. Serum-free culture medium was collected from equal numbers of infected cells, concentrated, and electrophoresed in a 12% SDS-PAGE gel containing 0.2% (w/v) casein with or without 5 μ g/mL plasminogen (*top*) and a 10% SDS-PAGE gel containing 0.1% (w/v) gelatin (*bottom*). Gels were incubated in the presence of 2.5% Triton X-100 for 2 h at room temperature and subsequently at 37°C overnight in a reaction buffer. Thereafter, gels were stained with 0.25% Coomassie blue and proteolysis was detected as a white band against a blue background.

siRNA. When c-Met is blocked, ERK-MAPK and the PI3K pathway, both known regulators of integrin transcription, are inhibited (4, 35). Our finding that both α_5 and β_1 integrin mRNA are inhibited agrees with studies showing that both α_5 and β_1 integrins

are regulated transcriptionally (35–37). The respective two promoters have similar transcription factor binding sites, including a proximal AP-1 and SP-1 site, and both lack a TATA and CCAAT box. The four other genes shown in this study to be regulated by

c-Met (*MMP-2*, *MMP-9*, and *urokinase* and *its receptor*) are also, at least in part, regulated transcriptionally through very similar regulatory mechanisms (38, 39). We and others have shown that c-Met can induce transcription from these binding sites through the ERK-MAPK pathway, raising the possibility that inhibition of c-Met inhibits the promoter of all these genes and consequently adhesion and invasion (40, 41). It also supports the idea that it might be more advantageous to inhibit a key signaling receptor that has a unique role in the regulation of many tumor-associated genes than to inhibit a single downstream protease whose function can be substituted by many other proteases.

Collectively, our findings are consistent with a model in which the adhesion and invasion of ovarian cancer cells is, at least partially, controlled by c-Met through the activation of $\alpha_5\beta_1$ integrin and tumor-associated proteases. We propose that the c-Met siRNA impairs peritoneal metastasis through the inhibition of those key signaling pathways and integrins that mediate peritoneal tumor adhesion. The cells that do succeed in attaching in these conditions have less protease activity at their disposal, which further hampers their ability to establish a viable metastatic focus. Furthermore,

there is evidence that proteases and their receptors collaborate with integrins to facilitate tumor cell adhesion and invasion. Binding of the urokinase receptor to $\alpha_5\beta_1$ integrin promotes migration on fibronectin and increases integrin-dependent adhesion of several tumor cell lines, including SKOV-3ip1 (42, 43). However, despite inhibition of c-Met, these cells form metastases that are similar in size and proliferation rate to those formed by control-treated cells. This model is also supported by our findings in human ovarian cancer tissue, where c-Met expression is similar in the primary ovarian tumor and corresponding metastases.

Our *in vivo* studies showed an 85% inhibition in the number of tumor nodules, tumor weight, and ascites in mice injected with the c-Met siRNA. Saga et al. targeted HGF/SF and stably transfected NK4, which is a HGF/SF fragment that competes with HGF/SF for binding to the c-Met receptor, into an ovarian cancer cell line. After *in vivo* injection, NK4 expression did not affect *in vivo* tumor growth. However, injection of the NK4 stably expressing cell line produced 70% fewer peritoneal metastases (11). These results together with ours support the idea that the HGF/SF-c-Met axis is important for ovarian cancer metastasis. The fact that, in ovarian

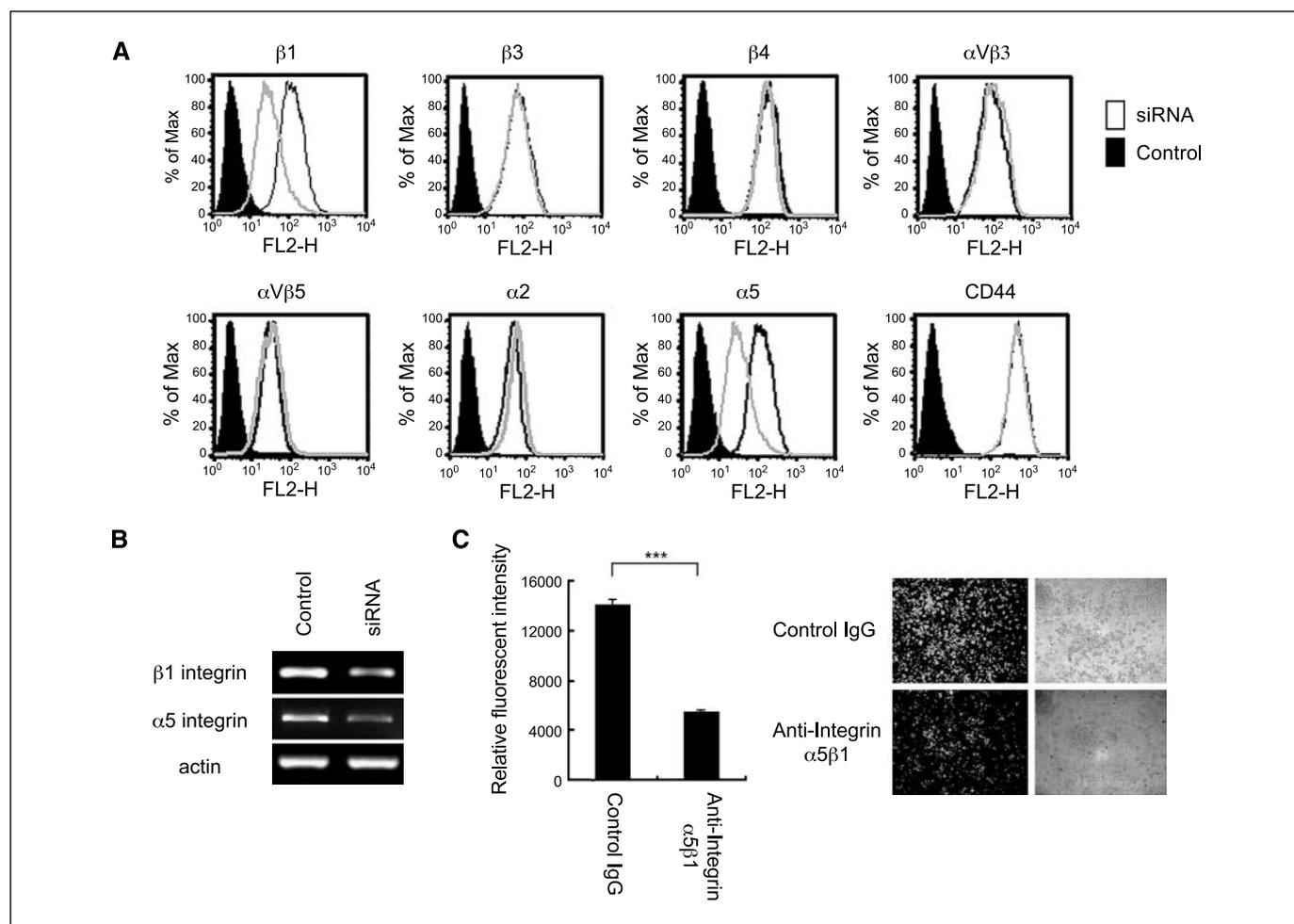


Figure 4. The c-Met siRNA suppresses the expression of β_1 and α_5 integrin. **A**, the surface expression of adhesion receptors was evaluated by fluorescence-activated cell sorting. SKOV-3ip1 cells were infected with control virus (black) or si-hMet-Ad5 (gray) and then cells were incubated with control IgG (filled histogram), primary α_2 , α_5 , β_1 , β_3 , β_4 , $\alpha V\beta_3$, and $\alpha V\beta_5$ integrin, or an antibody against CD44 followed by a secondary goat antimouse IgG conjugated with PE. **B**, RT-PCR. Total RNA was extracted from SKOV-3ip1 cells infected with control virus or si-hMet-Ad5, reverse transcribed, and amplified with α_5 and β_1 integrin-specific primer. Results are representative of three different experiments. **C**, blocking integrin $\alpha_5\beta_1$ decreases *in vitro* adhesion onto primary human mesothelial cells. SKOV-3ip1 cells were preincubated with integrin $\alpha_5\beta_1$ blocking antibody overnight, labeled with a fluorescent dye (CMTPIX), resuspended in serum-free medium, and plated onto 96-well plates coated by a monolayer of HPMCs. After incubation for 1 h at 37°C, plates were washed to discard nonadherent cells and the number of adherent cells was quantified by measuring fluorescence intensity. Columns, mean of three independent experiments. ***, $P < 0.001$.

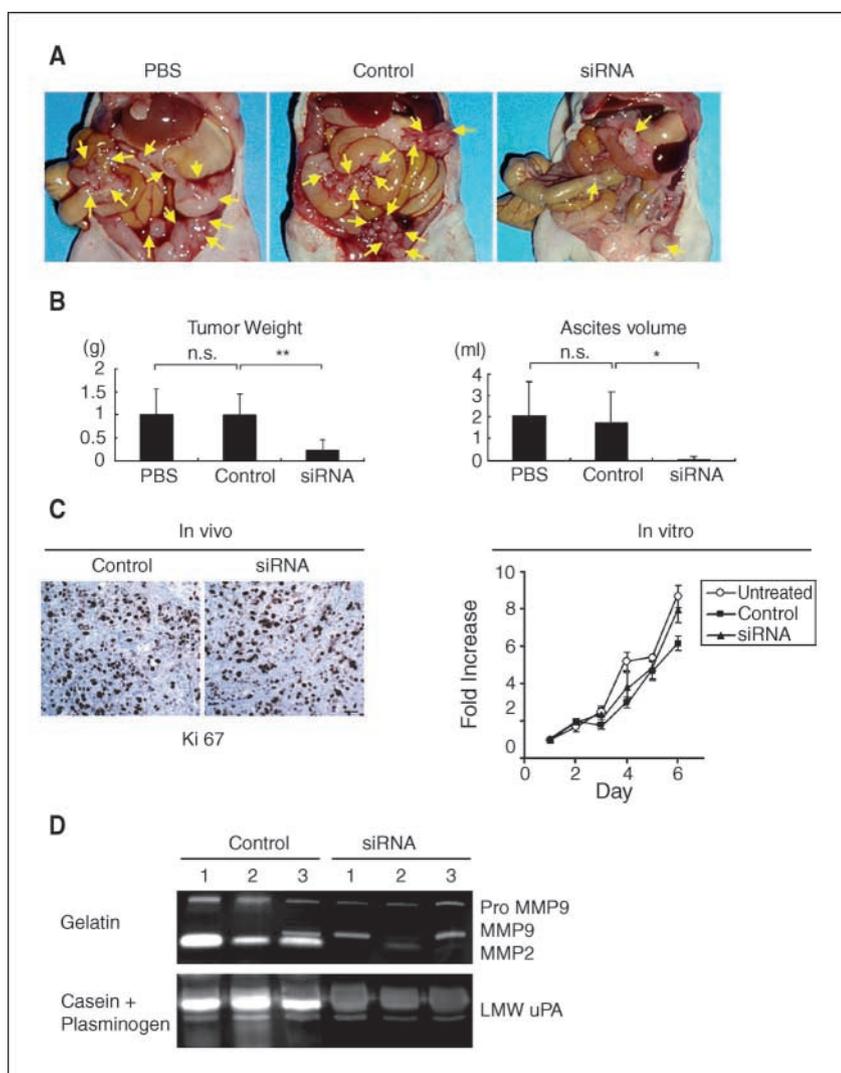


Figure 5. Effects of a c-Met siRNA on i.p. tumor burden, ascites, protease activity, and proliferation in mice inoculated with SKOV-3ip1 cells. SKOV-3ip1 cells (1.0×10^6) were suspended as single cells in a volume of 500 μ L PBS and injected i.p. One week after injection, the si-hMet-Ad5²²¹ (1.0×10^9 pfu/mouse), control adenovirus, or an equivalent volume of PBS was injected twice weekly for a total of 4 wks of treatment. **A**, representative views of the peritoneal cavity of mice treated with PBS alone, control adenovirus, or si-hMet-Ad5²²¹. Arrows, tumor. **B**, effects of Met siRNA on i.p. tumor weight and ascites formation ($n = 10$). At autopsy, tumors were excised and weighed, and ascites fluid was collected and measured. Columns, mean; bars, SD. *, $P < 0.05$, compared with the control group; **, $P < 0.01$, compared with the control group. **C**, left, representative areas of tumor in the mouse omentum were stained for the proliferation marker Ki-67. Bar, 100 μ m. Right, effects of Met RNAi on the proliferation of ovarian cancer cells *in vitro*. SKOV-3ip1 cells were infected with the control or the si-hMet-Ad5²²¹ adenovirus (siRNA) and proliferation was measured using a fluorescent dye that incorporates into nucleic acids. **D**, proteolytic activity in inoculated SKOV-3ip1 tumors. The inoculated tumors from three different mice ($n = 3$, each group) were excised and lysed and a gelatin (*top*) or a plasminogen zymogram (*bottom*) was done.

cancer, c-Met does not regulate tumor growth or angiogenesis, as is reported for other epithelial cancers (44, 45), emphasizes that the biology of ovarian cancer is different from that of hematogenously metastasizing cancers.

In summary, this study shows that c-Met is highly expressed in a subset of ovarian cancer patients and that its inhibition can reduce adhesion, invasion, metastasis, and ultimately tumor burden. It is tempting to speculate that, after the optimal surgical debulking of a patient with ovarian cancer, consolidation therapy with a drug that targets metastasis will delay the repopulation of the peritoneal cavity by ovarian cancer cells.

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c-Met Overexpression Is a Prognostic Factor in Ovarian Cancer and an Effective Target for Inhibition of Peritoneal Dissemination and Invasion

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