Mesothelial cells promote early ovarian cancer metastasis through fibronectin secretion

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Ovarian cancer (OvCa) metastasizes to organs in the abdominal cavity, such as the omentum, which are covered by a single layer of mesothelial cells. Mesothelial cells are generally thought to be “bystanders” to the metastatic process and simply displaced by OvCa cells to access the submesothelial extracellular matrix. Here, using organotypic 3D cultures, we found that primary human mesothelial cells secrete fibronectin in the presence of OvCa cells. Moreover, we evaluated the tumor stroma of 108 human omental metastases and determined that fibronectin was consistently overexpressed in these patients. Blocking fibronectin production in primary mesothelial cells in vitro or in murine models, either genetically (fibronectin 1 floxed mouse model) or via siRNA, decreased adhesion, invasion, proliferation, and metastasis of OvCa cells. Using a coculture model, we determined that OvCa cells secrete TGF-β1, which in turn activates a TGF-β receptor/RAC1/SMAD-dependent signaling pathway in the mesothelial cells that promotes a mesenchymal phenotype and transcriptional upregulation of fibronectin. Additionally, blocking α5 or β1 integrin function with antibodies reduced metastasis in an orthotopic preclinical model of OvCa metastasis. These findings indicate that cancer-associated mesothelial cells promote colonization during the initial steps of OvCa metastasis and suggest that mesothelial cells actively contribute to metastasis.
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Introduction

The biology of serous high-grade ovarian cancer (OvCa) is different from that of most other solid tumors, since OvCa is predominantly confined within the abdominal and pleural cavities and rarely metastasizes hematogenously (1). Moreover, OvCa is generally only superficially invasive, although advanced disease is characterized by large intra-abdominal tumors in the ovary and the omentum. During OvCa dissemination, the cancer cells detach from the primary site, which can be the fallopian tube, the ovary, or the peritoneum. Subsequently, the peritoneal fluid carries the OvCa cells to secondary sites of implantation, including the omentum, the most common site of OvCa metastasis. These sites are exclusively organs with a single layer of mesothelial cells covering an underlying stroma composed of extracellular matrices (ECM) and stromal cells (2, 3). Consequently, OvCa cells must invade through the barrier of mesothelial cells on the peritoneum, omentum, and bowel serosa to effectively form metastases.

Mesothelial cells were originally depicted as a mechanical barrier that must be pushed to the side by tumor cells (4, 5). In coculture, cancer cells induced human mesothelial cells to retract from the peritoneum and omentum, thereby exposing the underlying ECM (4). Iwanicki and colleagues extended these findings by showing that OvCa spheroids use myosin-generated force to clear mesothelial cells in human mesothelial cell line monolayers (5, 6). Tumor-induced apoptosis may also be important for mesothelial cell clearance and peritoneal invasion (7).

However, reports that mesothelial cells may induce the motility of OvCa cells supports a possible tumor-promoting role for these cells during OvCa metastasis. Rieppi et al. revealed that conditioned media (CM) of primary human mesothelial cells induced migration of OvCa cell lines through a gelatin-coated Boyden chamber (8), and a later paper demonstrated that mesothelial cells promote OvCa adhesion (9). Collectively, these findings were the first evidence that mesothelial cells actively participate in the establishment of the OvCa metastatic niche. This concept is consistent with the observation that cancer cells recruit local stromal cells to promote and stabilize their growth (10). The interaction between cancer and stromal cells has primarily been studied in cancer-associated fibroblasts (CAFs), which have been shown to promote almost every aspect of local tumor growth (11). In the OvCa microenvironment, CAFs (12, 13) and cancer-associated adipocytes (14, 15) promote invasion and metastasis, which indicates that OvCa cells have the capability to recruit various types of stromal cells. It is therefore unlikely that mesothelial cells are simply “bystanders” that must be pushed out of the way by invading OvCa cells in the metastatic process. Rather, it is likely that they are recruited by OvCa cells and reprogrammed to facilitate tumor growth. Indeed, cancer cell CM may stimulate mesothelial cell motility (16, 17).
Increased expression of fibronectin (encoded by FN1), a key component of the ECM, has been detected in OvCa metastases compared with the primary ovarian tumor (14, 18), and fibronectin is also present in ascites (19). While multiple integrins bind fibronectin at specific amino acid sequences (i.e., PHSRN and RGD), αβ1 integrin is selective for fibronectin (20). The binding of integrins to the ECM promotes integrin clustering and subsequently triggers integrin-mediated intracellular signal transduction (21), while at the same time, integrin receptors mediate fibronectin matrix assembly (22). Functionally, fibronectin induces adhesion, proliferation, and invasion of tumor cells and contributes to the formation, adhesion, and disaggregation of OvCa spheroids (16, 23, 24).

Given our previous finding that fibronectin is overexpressed in OvCa metastases (14), as well as indications that mesothelial cells might communicate with OvCa cells, we hypothesized that OvCa-induced fibronectin expression by mesothelial cells may play a critical role in the early steps of OvCa metastasis. We report here that OvCa cells stimulated mesothelial cells to produce fibronectin through secretion of TGF-β1, thereby activating a TGF-β receptor 1 (TGF-βRI)/RAC1/SMAD-dependent signaling pathway in the mesothelial cells. The activated mesothelial cells promoted metastasis by supporting tumor cell adhesion, invasion, and proliferation.

**Results**

**OvCa cells induce fibronectin production early in metastasis to the human omentum.** The most common site of OvCa metastasis in patients with serous high-grade cancers is the omentum. In fact, in mouse models, OvCa cells home to the omentum immediate- ly after i.p. injection (14). In a previous study, we used a reverse phase protein array to identify protein expression in paired ovarian and omental metastases and found that fibronectin expression was higher in the omental metastasis than in the primary tumor, while other ECM proteins, such as vitronectin, were more highly expressed in the primary tumor (14). This was confirmed by immunoblot using snap-frozen tissue of the paired samples from the same cohort of patients (Supplemental Figure 1A and Supplemental Methods; supplemental material available online with this article; doi:10.1172/JCI74778DS1). The results were further validated using a tissue microarray (TMA) constructed with tissue from omental metastases from 108 patients with advanced OvCa. In 93% of these patients (100 of 108), fibronectin expression was much stronger in the stromal than in the epithelial cell compartment (Figure 1A). To determine whether fibronectin expression is an early or late event during omental/peritoneal metastasis, we identified samples from a small subgroup of women diagnosed with stage IIIA serous-papillary OvCa (FIGO 1988 staging), defined by microscopic disease to the omentum (Supplemental Figure 1B), which allowed us to study early OvCa metastasis. Immunohistochemical staining showed that fibronectin expression was induced below proliferating metastatic cells that had not yet invaded the basement membrane (Figure 1B). Once the tumor invaded, the stroma around the serous tumor islands had very strong fibronectin expression (Figure 1B), confirming the TMA results. Direct comparison of protein and RNA levels of fibronectin in normal omental and omental metastatic tissues showed an increased level of fibronectin in the omental tumor harboring OvCa metastases (Figure 1C and Supplemental Figure 1C). In addition, fibronectin was abundant in the ascites of OvCa patients (Supplemental Figure 1D and ref. 19).

To determine whether the fibronectin expression in metastatic OvCa is of functional significance, ECM was extracted from unfixed benign and tumor transformed omental tissue removed at surgery from separate patients. In the ECM from omental metastasis, fibronectin expression was found to be higher, and multiple cleavage forms of fibronectin were detected (Figure 1D). Compared with ECM from normal omentum, 2 OvCa cell lines, HeyA8 and SKOV3ip1, showed increased adhesion and proliferation to the omental ECM extracted from metastatic tumors (Figure 1E and Supplemental Figure 1E). Cells bind to fibronectin through specific cell surface integrins, including the fibronectin receptor αβ1 integrin and the vitronectin receptor α3β1 integrin (21). Adhesion and proliferation of OvCa cells to tumor-derived ECM could be abrogated using blocking antibodies against these integrins as well as an RGD peptide, whereas control IgG, the control RAD peptide, and a β1 integrin antibody had no inhibitory effect (Figure 1E and Supplemental Figure 1E).

Next, we investigated whether early metastasis of OvCa cells to the omentum could be modeled ex vivo with human omental tissue and in vivo in mice. Injection of HeyA8 OvCa cells i.p. induced strong expression of fibronectin in the mouse omentum (Figure 2A). Attachment of fluorescently labeled OvCa cells to a piece of human omentum followed by fluorescence-activated cell sorting (FACS) to separate OvCa and omental cells also showed strong protein and RNA induction of fibronectin in the surface cells that interacted with the cancer cells, whereas expression in the cancer cells was unchanged (Figure 2B).

The mesothelium covers all organs within the abdominal cavity, including the peritoneum and the omentum. It is composed of a monolayer of mesothelial cells, on top of an ECM interspersed with resting fibroblasts that are responsible for secreting most of the ECM in the basement membrane (Figure 1B, Supplemental Figure 1B, and refs. 25, 26). We and others have modeled the mesothelium in a 3D culture (5, 25) using primary human omental fibroblasts and mesothelial cells (Supplemental Figure 2). Co-culture of SKOV3ip1 cells with the organotypic 3D culture induced fibronectin synthesis and matrix assembly (Figure 2C). Fibronectin matrix secretion and assembly can be assessed by detecting the conversion of cell-associated, deoxycholic acid (DOC)-soluble fibronectin fibrils into a DOC-insoluble fibril network (22). Co-culture of OvCa cells with the 3D culture for 48 hours induced the secretion of soluble fibronectin in the ECM of the 3D culture and also induced the aggregation of fibronectin as a dense DOC-insoluble matrix (Figure 2C). Moreover, binding of inactive fibronectin dimers to integrins on the OvCa cell surface induced a dense fibronectin matrix (Figure 2D). These data suggest that binding of OvCa cells to the omentum induces early fibronectin production and matrix assembly, which is functionally important for adhesion, migration, and invasion.

**OvCa cells stimulate fibronectin expression in mesothelial cells.** Since mesothelial cells are the first cell type to interact with metastasizing OvCa cells (27), we sought to determine how the interaction between OvCa cells and mesothelial cells results in...
Figure 1. Fibronectin is overexpressed in the stroma of omental metastases. (A) Immunohistochemistry for fibronectin (FN) levels in the tumor and stromal compartments of omental metastases (n = 108) was analyzed in tumor sample cores using Aperio ImageScope and Spectrum software (see Supplemental Figure 9). Black dots, outliers; boxes, interquartile range (IQR); lines within boxes, median. ***P < 0.001, Wilcoxon rank test (median ± 1.5 IQR). 3 different tumor tissue cores from separate patients are shown. (B) Immunohistochemistry for fibronectin in tissue from a patient coincidentally detected with early, microscopic OvCa metastasis to the omentum (stage IIIA; representative sections of affected areas are shown). Arrowhead, mesothelial cells; arrows, OvCa cells. (C) Immunohistochemistry for fibronectin expression in omental tissues (n = 11) sampled from patients treated for benign disease and omental metastases (n = 43) removed from patients with serous papillary OvCa (mean ± SEM). *P < 0.05. (D) Immunoblot analysis of ECM extracted from omental tissues (n = 3) sampled from patients treated for benign disease and omental metastases removed from patients with serous papillary OvCa. rh-FN, recombinant human fibronectin. (E) Left: Adhesion (30 minutes) of OvCa lines SKOV3ip1 and HeyA8 to the isolated ECM (mean ± SEM; n = 5; 3 independent experiments). mlgG, murine IgG control. *P < 0.05. Student’s t test. Scale bars: 100 μm.
Figure 2. OvCa cells induce fibronectin matrix assembly in stromal cells. (A) Fibronectin protein expression in full mouse omentum after in vivo i.p. injection of HeyA8 OvCa cells for 48 hours. Immunoblot analysis was performed using a fibronectin-specific antibody. (B) Left: Ex vivo full human omentum adhesion, invasion, and proliferation assays with fluorescently labeled OvCa cells. Middle: Immunoblot analysis of fibronectin protein in the surface cells of the human omentum cultured alone (unbound) or with fluorescently labeled SKOV3ip1 cells (bound; after FACS). OD of fibronectin is shown normalized to GAPDH signal. Right: qRT-PCR analysis of FN1 mRNA expression. qRT-PCR was performed on mRNA using fibronectin specific probes (mean ± SEM; n = 5; 3 independent experiments). *P < 0.05, Student’s t test. (C) Fibronectin protein production and matrix secretion were analyzed in the 3D omental culture cocultured with SKOV3ip1 cells. Left: IF was performed using a fibronectin-specific antibody, and fibronectin production (green; IF with Triton-X 100) and fibronectin matrix secretion (red; IF without Triton-X 100) were analyzed. Right: Immunoblot analysis of fibronectin matrix production using DOC-soluble and -insoluble fractions of fibronectin (indicating a dense matrix) in SKOV3ip1 cells, 3D culture, or coculture. (D) Fibronectin matrix assembly by OvCa cells cultured on fibronectin. Fibronectin was seeded on a glass-bottomed plate, and SKOV3ip1, HeyA8, or HT1080 cells were added for 24 hours. IF for fibronectin (red) was performed; the formation of a dense fibronectin matrix was indicated by presence of fibrils. Scale bars: 50 μm.
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(Figure 3C). Fibronectin induction in mesothelial cells was not limited to direct OvCa cell/mesothelial cell interaction, since CM from the OvCa cells was able to induce both protein and mRNA expression of fibronectin (Figure 3D). CM from the high-grade serous OvCa cell lines Kuramochi and Tyk-nu, as well as from RKO colon cancer cells, induced fibronectin in mesothelial cells (Supplemental Figure 3, A and B). The mesothelial cell response to cancer cells was not limited to omental tissue–derived mesothelial cells. Both peritoneal wall– and fallopian serosa–derived mesothelial cells increased fibronectin production when treated with OvCa cell CM (Supplemental Figure 3, C and D).

Next, we inhibited fibronectin production (Figure 4A) and secretion (Supplemental Figure 4 and Supplemental Methods) fibronectin production. Fluorescently labeled OvCa cells were cocultured with primary human mesothelial cells for 48 hours, then separated by FACS. The cocultured OvCa cell lines strongly induced both intracellular and secreted fibronectin in the mesothelial cells, which laid down a dense fibrillar fibronectin matrix, whereas the cell lines cultured alone secreted a negligible amount of fibronectin (Figure 3, A and B). The OvCa cell–mediated fibronectin induction was paralleled by FN1 mRNA induction in the mesothelial cells (Figure 3C), suggestive of transcriptional regulation of fibronectin upon interaction with the OvCa cells. Indeed, transfection of primary human mesothelial cells with the full-length FN1 promoter (28), followed by cocultivation with SKOV3ip1 cells, increased FN1 promoter activity almost 40-fold (Figure 3C). Fibronectin induction in mesothelial cells was not limited to direct OvCa cell/mesothelial cell interaction, since CM from the OvCa cells was able to induce both protein and mRNA expression of fibronectin (Figure 3D). CM from the high-grade serous OvCa cell lines Kuramochi and Tyk-nu, as well as from RKO colon cancer cells, induced fibronectin in mesothelial cells (Supplemental Figure 3, A and B). The mesothelial cell response to cancer cells was not limited to omental tissue–derived mesothelial cells. Both peritoneal wall– and fallopian serosa–derived mesothelial cells increased fibronectin production when treated with OvCa cell CM (Supplemental Figure 3, C and D).
in primary human mesothelial cells using siRNA and evaluated adhesion, invasion, and proliferation of 2 OvCa cell lines. Without the fibronectin support from the mesothelial cells, neither of the OvCa cell lines were able to adhere (59% of control), invade (36%), or proliferate (54%) as efficiently (Figure 4A). Furthermore, when fibronectin was knocked down in the mesothelial cells that covered a full piece of human omentum, the OvCa cells were again not able to adhere, invade, or proliferate as efficiently as control transfected cells (Figure 4B).

These experiments were corroborated in vivo using a transgenic mouse model of fibronectin deficiency. Homozygous floxed fibronectin 1 (Fn1) mice (Fn1fl/fl mice) do not have an obviously abnormal phenotype, and have normal fertility and life spans. However, upon induction of ischemic brain injury, the deletion of Fn1 leads to large brain infarcts, which are, at least in part, due to the absence of the antiapoptotic function of fibronectin (29). Using the Fn1fl/fl mice, we endeavored to determine the role of fibronectin during early metastasis in vitro and in vivo. Primary resting omental mouse mesothelial cells established from Fn1fl/fl mice were infected with Cre recombinase–containing adenovirus (referred to herein as Ad-Cre) to delete Fn1. Because resting mouse mesothelial cells (Figure 2A), like their human counterparts (Figure 3A), did not constitutively express fibronectin, they were stimulated with TGF-β, a known inducer of fibronectin expression (30). Deletion of fibronectin abrogated TGF-β-mediated Fn1 mRNA induction (Figure 5A and Supplemental Figure 5), confirming successful Fn1 deletion. When the primary Fn1fl/fl mesothelial cells were cocultured with ID8 mouse OvCa cells stably expressing GFP, adhesion, invasion, and proliferation were significantly impaired, at levels 60%, 60%, and 74%, respectively, of the empty adenovirus control (Ad-Cont) (Figure 5A). Next, we deleted fibronectin expression in vivo on the surface cells lining the abdominal cavity through i.p. injection of Ad-Cre into Fn1fl/fl mice. Since the abdominal cavity and organs are covered by mesothelial cells (2, 25, 27), i.p. injection of Ad-Cre will delete fibronectin expression, primarily in the mesothelial cells. Quantitative real-time RT-PCR (qRT-PCR) analysis 2 days after Ad-Cre injection showed successful in vivo knockdown of fibronectin (Figure 5B). Compared with Ad-Cont–injected mice, Ad-Cre–pretreated Fn1fl/fl mice infected i.p. with GFP-expressing ID8 mouse OvCa cells yielded fewer omental metastases (Figure 5, C and D). Furthermore, treatment of GFP-expressing ID8 cells with an α5 integrin–specific antibody inhibited OvCa metastasis by 52% in the Fn1fl/fl mice, while knocking out fibronectin with Ad-Cre treatment in the Fn1fl/fl mice reduced OvCa metastasis by 76%. Combination therapy with α5 integrin antibody and Ad-Cre treatment in the Fn1fl/fl mice inhibited OvCa metastasis by 86%. These data suggest that the prometa-static function of fibronectin involves a non-fibronectin-receptor-mediated factor. On the basis of the combined data in Figures 4 and 5, we propose that mesothelial cell fibronectin expression promotes early OvCa metastasis.

**Tumor cells induce fibronectin expression in mesothelial cells through a TGFβR1/RAC1/SMAD3–dependent signaling pathway.** Analysis of gene expression data from the Australian Ovarian Cancer Study (AOCs) and from the Cancer Genome Atlas (TCGA) OvCa data showed that FNI mRNA was most highly expressed in the C1 molecular subtype (high stromal response; Supplemental

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**Figure 4. Inhibition of fibronectin in mesothelial cells or omentum impairs OvCa cell adhesion, invasion, and proliferation.** (A) Fibronectin knockdown in primary human mesothelial cells. Immunoblot analysis of fibronectin in primary human mesothelial cells transfected with fibronectin-specific (FN), vitronectin-specific (Vn), or control (NC) siRNA. Functional assays investigating the effect of fibronectin knockdown in mesothelial cells on OvCa cell adhesion (30 minutes), invasion (24 hours), and proliferation (96 hours). Primary human mesothelial cells were transfected with fibronectin-targeted or control siRNA, followed by addition of fluorescently labeled SKOV3ip1 and HeyA8 OvCa cells, which were detected using a fluorescence reader (mean ± SEM; n = 5 [adhesion and proliferation], 3 [invasion]; 3 independent experiments). *P < 0.05, Student’s t test. (B) Fibronectin knockdown in pieces of full human omentum. Left: Transfection of fibronectin siRNA (specifically designed for in vivo use) in pieces of full human omentum (72 hours); FNI mRNA downregulation was confirmed in detached surface cells (after scraping off surface cells of the omentum) using qRT-PCR. Right and bottom: Adhesion, invasion, and proliferation of OvCa cells was inhibited on full human omentum when fibronectin expression in omental surface cells decreased (mean ± SEM; n = 5; 3 independent experiments). *P < 0.05, Student’s t test.
Figure 5. Genetic knockdown of Fn1 reduces metastasis. (A) Left: qRT-PCR analysis for Fn1 on mRNA isolated from mouse mesothelial cells cultured from Fn1fl/fl mice treated for 72 hours with Ad-Cont or Ad-Cre, followed by treatment with TGF-β1 for 24 hours. Right: Functional assays investigating the effect of fibronectin knockdown in primary Fn1fl/fl mesothelial cells on ID8 mouse OvCa cell adhesion, invasion, and proliferation. (B–D) In vivo knockdown of fibronectin in omental surface cells of Fn1fl/fl mice. Ad-Cre or Ad-Cont was injected i.p. into Fn1fl/fl mice for 96 hours to downregulate fibronectin, followed by i.p. injection of 4 × 10⁶ GFP-expressing ID8 OvCa cells. (B) 48 hours after injection, the mouse omentum was removed and digested, omental surface cells were collected by FACS, and qRT-PCR was performed for Fn1 on mRNA isolated from the cells. (C and D) Metastasis assay. (C) Fn1fl/fl or control C57BL/6 mice were injected with PBS, Ad-Cre, or Ad-Cont. (D) GFP-expressing ID8 cells were mixed with control IgG or 10 μg/ml murine α5 integrin antibody for 15 minutes prior to i.p. injection. 72 hours after ID8 cell injections, the mouse omentum was removed, imaged using fluorescent microscopy (right), and digested, and cancer cells were quantified using fluorescent microscopy (left). Data are mean ± SEM (n = 3 [invasion and qRT-PCR assays] or 5 [other assays]). *P < 0.05, Student’s t test. Scale bars: 2 mm.
Figure 6A), which is associated with serous high-grade cancer and the poorest survival (31). Subsequent pathway analysis indicated that the epithelial to mesenchymal transition (EMT) was the second most significant biological pathway that correlated with elevated fibronectin expression in the AOCs and TCGA cohorts (Supplemental Tables 1 and 2 and ref. 31). This bioinformatic analysis was then tested experimentally. Addition of SKOV3ip1 CM, or coculture of the cancer cells with primary mesothelial cells, led to decreased E-cadherin and increased vimentin protein and mRNA expression in the mesothelial cells (Figure 6A and Supplemental Figure 6B). Additionally, OvCa CM induces EMT morphology in the mesothelial cells (Supplemental Figure 6, C and D, and ref. 32). We conjectured that cancer cell–derived TGF-β1 mediates fibronectin production and/or secretion in mesothelial cells, because (a) TGF-β1–dependent EMT pathways correlated with fibronectin expression (Supplemental Table 2), (b) TGF-β1 induced EMT in mesothelial cells (Supplemental Figure 6, C and D, and ref. 33), (c) SKOV3ip1 cells secreted TGF-β (Supplemental Figure 6E), and (d) TGF-β1 is highly expressed in omental metastasis (34). To test this hypothesis, TGF-β1 expression was inhibited in SKOV3ip1 cells using siRNA. CM from these cells induced less fibronectin production in mesothelial cells than did CM from control siRNA–stimulated cells (Figure 6B and Supplemental Figure 6F). SKOV3ip1 CM–stimulated mesothelial cells were then treated with an inhibitor of TGF-βRI, an antibody against TGF-βRII, and inhibitors of small G proteins (the downstream TGF-β1 effectors RAC, RAS, and RHO; ref. 35). The TGF-βRI inhibitor, the TGF-βRII antibody, and the RAC inhibitor blocked fibronectin secretion by mesothelial cells; however, the RAS and RHO inhibitors had no such effect (Figure 6C). Comparably, FNI mRNA expression was abrogated with RAC1 inhibitor treatment in SKOV3ip1 cocultured mesothelial cells (Supplemental Figure 7A). Consistent with a prominent role for TGF-β1 signaling in mesothelial cells, coculture of the 2 cell types showed strong induction of phosphorylated SMAD2/3 (36) in mesothelial cells, as exhibited by immunoblot after FACS and immunofluorescence (IF) (Figure 7A). When we inhibited SMAD3 using siRNA in mesothelial cells or added a TGF-βRII inhibitor or TGF-βRII antibody to mesothelial cells followed by the addition of SKOV3ip1 CM, fibronectin secretion was impaired (Figure 7B), which was paralleled by reduced FNI mRNA in the mesothelial cells (Supplemental Figure 7A).

Coculture of OvCa cells with mesothelial cells strongly induced RAC1 activity, while total RAC1 expression was unchanged (Figure 7C and Supplemental Figure 7B). The increase in RAC1–GTP was specifically observed (by IF) in mesothelial cells after coculture with SKOV3ip1 cells (Figure 7C). Inhibition of RAC1 expression in mesothelial cells with either a RAC1 siRNA or the RAC1 inhibitor abrogated FNI mRNA in cocultured mesothelial cells and fibronectin secretion in OvCa cell CM–stimulated mesothelial cells (Figure 7D and Supplemental Figure 7A). Because our findings suggested that both RAC1 and TGF-β1 signaling regulate fibronectin expression, we asked whether TGF-β1 regulates fibronectin through RAC1 signaling. Indeed, the TGF-β1–mediated induction of fibronectin expression in mesothelial cells could be abrogated by blocking RAC1 signaling (Figure 7E). Taken together, these data suggest that tumor cells induce EMT in mesothelial cells through TGF-β1 signaling.

Blocking α5 integrin or β1 integrin prevents OvCa metastasis. In view of the critical role of fibronectin during early OvCa metastasis, 2 potential clinically applicable fibronectin receptor function blocking antibodies were evaluated in vitro and in vivo. The established OvCa cell lines HeyA8 and SKOV3ip1 were fluorescein labeled and then cultured on the 3D culture with antibodies. Both α5 integrin (37) and β1 integrin (38) blocking antibodies prevented...
testing, ATN-161 (Ac-PHSCN-NH2), a 5-mer capped peptide derived from the fibronectin synergy region, was evaluated in vitro and in vivo. The synergy region of fibronectin is a ligand for $\alpha_5\beta_1$ integrin, which increases the avidity of $\alpha_5\beta_1$ integrin for the fibronectin RGD sequence (19). The peptide blocked both proliferation and invasion of the established OvCa cell lines HeyA8 and SKOV3ip1 by an average of 29% and 52%, respectively (Supplemental Figure 8A). To study the in vivo efficacy of ATN-161, we took 2 approaches (37): (a) testing the peptide in a prevention study in vitro adhesion by an average of 60% and invasion and proliferation by an average of 40% (Figure 8A). To study the in vivo efficacy of the 2 antibodies, we tested them in a prevention study (treatment the day of and 2 and 4 days after OvCa cell intravarian injection) that mimicked treatment during initiation of metastasis. Pretreatment of mice with $\alpha_5$ integrin or $\beta_1$ integrin blocking antibodies reduced metastasis number and tumor weight by more than 90% compared with IgG control–treated mice (Figure 8B). Finally, the therapeutic effect of a compound now in clinical testing, ATN-161 (Ac-PHSCN-NH2), a 5-mer capped peptide derived from the fibronectin synergy region, was evaluated in vitro and in vivo. The synergy region of fibronectin is a ligand for $\alpha_5\beta_1$ integrin, which increases the avidity of $\alpha_5\beta_1$ integrin for the fibronectin RGD sequence (19). The peptide blocked both proliferation and invasion of the established OvCa cell lines HeyA8 and SKOV3ip1 by an average of 29% and 52%, respectively (Supplemental Figure 8A). To study the in vivo efficacy of ATN-161, we took 2 approaches (37): (a) testing the peptide in a prevention study...
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of abdominally metastasizing tumors remains poorly defined.
The peritoneal cavity and the omentum are lined by the meso-
ethelium, a monolayer of mesothelial cells resting on a basement
membrane. This tissue functions to maintain serosal integrity and
provide a nonadhesive surface that allows smooth bowel move-
ments. It also acts as a permeability barrier, secreting various sub-
stances involved in the regulation of peritoneal permeability and
local host defense (2). By testing several stromal cell components
using an organotypic 3D model of the human omentum/perito-
neum that we developed previously (25, 39), we found here that
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sion, migration, and invasion in vitro (Figure 4), and by using an

treatment the day of and 3 and 5 days after OvCa cell i.p. injection)
that mimicked treatment of microscopic disease, and (b) treating
advanced tumors in an intervention study (treatment 3 times per
week starting on day 7 after OvCa cell i.p. injection), a design that
mimicked treatment of advanced disease. Pretreatment of mice
with ATN-161 in the prevention study reduced metastasis number
by 52% and tumor weight by 70% compared with control-treated
mice (Supplemental Figure 8B). Surprisingly, the peptide also
reduced metastatic spread by 66% and tumor weight by 65% in
the intervention study, when the drug was given after the establish-
ment of tumors (Supplemental Figure 8C). Taken together, these
data suggest that inhibiting the interaction of integrins with fibro-
nectin blocks both early and late steps of OvCa metastasis.

Discussion
In recent years, we have come to understand that stromal cells in
the tumor microenvironment and the ECM are important com-
ponents of the metastatic process (10). However, the role of these
cells in the molecular events underlying the early dissemination
of abdominally metastasizing tumors remains poorly defined.
The peritoneal cavity and the omentum are lined by the meso-
ethelium, a monolayer of mesothelial cells resting on a basement
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sion, migration, and invasion in vitro (Figure 4), and by using an
inducible floxed fibronectin mouse model, we reduced metasta-
sis in vivo (Figure 5). These results suggest that OvCa cells recruit
mesothelial cells, inducing them to secrete fibronectin to help the
cancer cells establish the initial metastatic colony (Figure 8C).
Indeed, the behavior of the primary mesothelial cells we studied
suggested that they become associated with cancer cells (i.e.,
cancer-associated mesothelial cells [CAMs]) in a manner ana-
gous to CAFs (40) and cancer-associated adipocytes (15, 41). This
supports a broader concept that cancer cells are able to recruit a
variety of cell types in the microenvironment to create an active-
ated tumor stroma that supports growth and metastasis. More
than 20% of OvCAs present as stage IV with pleural effusion, but
without radiologic evidence of intrapulmonary nodules. Although
we did not study pleural metastasis, it is interesting to note that
the pleural cavity is also lined by a single layer of mesothelial cells.
This raises the possibility that a similar mechanism contributes to
colonization of the pleura in OvCa.

In the presence of OvCa cells secreting TGF-β1, mesothelial
cells underwent EMT, upregulating vimentin and downregulating
E-cadherin. Their phenotype then changed from a cobble-
stone pattern to one of spindle-like cells resembling fibroblasts
(Figures 6 and 7). Secretion of fibronectin is a well-known mes-
enchymal marker that was previously used to confirm EMT in
mesothelial cells (2, 42). In mesothelial cells cocultured with
OvCa cells, TGF-β1 bound to the TGF-βRI and activated a RAC1/
SMAD-dependent signaling pathway that strongly induced the
transcriptional upregulation of fibronectin (Figures 6 and 7), thus
promoting the metastatic cascade. A similar mechanism has also
been described for keratinocytes, in which TGF-β1 downregu-
lates E-cadherin and upregulates a RAC-dependent signaling
pathway (43, 44). This is consistent with our present results that
RAC1 activity was upregulated when mesothelial cells and OvCa
cells were cocultured.

Activation of an EMT program, a general physiological
response of the mesothelium to stress, is a phenomenon reported
during peritoneal dialysis and peritoneal inflammation. Peritoneal
irritation caused by peritoneal dialysis induces mesothelial cells to
transition from an epithelial to a mesenchymal phenotype, a pro-
cess mediated by TGF-β1 (53). Chronic peritoneal dialysis induces
peritoneal fibrosis, which coincides with the EMT of mesothelial
cells and the loss of peritoneal membrane integrity and, conse-
quently, the loss of the ability of the membrane to act as a filter
during peritoneal dialysis (2). That mesothelial cells react to both
peritoneal dialysis and metastasis with EMT implies that the
response of mesothelial cells to cancer cells is not tumor cell spe-
cific, but is a generalized reaction of the peritoneum to irritants.
Mesothelial cells (45) secrete plasminogen activator inhibitors
(PAIs), urokinase (uPA), and VEGF upon local stress (e.g., dialysis
and infection). While this has not been directly studied in cancer,
it is probable that mesothelial cell secretion of these factors sup-
ports metastasis in a manner similar to that described here for
their secretion of fibronectin.

Availability of fibronectin to the cancer cells is a critical compo-
nent for metastasis. This is evidenced by a significant reduction in
metastasis or in the ability of cancer cells to invade if fibronectin is
knocked out in the peritoneal microenvironment (Figures 4 and 5).
Fibronectin has a well-established role in cancer metastasis, pro-
moting almost every aspect of tumor progression through activa-
tion of mitogenic signaling. In addition, it has known antiapoptot-
ic functions (29), promotes angiogenesis (21), and promotes the
recruitment of regulatory T cells (46). In invasive ovarian tumors,
fibronectin expression is found in the ECM, the submesothelial
basement membrane of omental metastasis (Figure 1), and in
ascites (19) and is associated with an adverse clinical prognosis
(47). Moreover, the cleavage of fibronectin into small fragments
by MMP-2 has been found to enhance the adhesion of OvCa cells
to the peritoneal surface (39). Taken together, these data clearly
indicate that fibronectin levels and proteolytic cleavage forms pro-
mote the aggressiveness of OvCa cells.

The concept presented here, that the fibronectin secreted by
mesothelial cells is important for the metastatic process of OvCa
cells, should have considerable relevance when selecting clini-
cally useful targeted therapies. We found that functionally block-
ing both α5 integrin and β3 integrin function inhibited the initial
metastasis of OvCa cells and reduced the initiation and growth of
metastatic tumors (Figure 8 and refs. 48, 49). Furthermore,
ATN-161, a short fibronectin peptide binding to α5β3 integrins,
blocked the initial metastasis of OvCa cells and inhibited the
growth of established tumors (Supplemental Figure 8). ATN-
161 was previously tested in preclinical models of breast and lung
cancer (34) and in a clinical phase I study that resulted in patients
with solid tumors showing stable disease (36). Our present data
suggest that maintenance therapy aimed at blocking the interac-
tion of fibronectin with the fibronectin receptor α5β3 integrin after
the end of adjuvant chemotherapy might delay tumor recurrence
and should be considered for further clinical testing. In addition,
knocking down fibronectin in mesothelial cells was significantly
more efficient in inhibiting OvCa metastasis than solely blocking
α5β3 integrin, which suggests that other integrin and nonintegrin
fibronectin receptors contribute to fibronectin-mediated metasta-
sis within the abdominal cavity.

On the basis of our combined data, we propose that the
interaction of OvCa cells with the mesothelium is an important
functional predeterminant of tumor metastasis and growth (Fig-
ure 8C). Is it possible, however, to reconcile the observation that
OvCa cells displaced mesothelial cells and induced their apop-
tosis with the finding that mesothelial cells promoted cancer cell
metastasis? It may be that the very initial interaction of OvCa
cells with mesothelial cells leads to displacement of some of the
mesothelial cells, allowing the OvCa cells to adhere directly to the
submesothelial ECM (6, 27, 32). The data presented here indicate
that after establishing their initial foothold, OvCa cells actively
recruit and activate adjacent mesothelial cells. Therefore, the
mesothelial cells play a key supporting role in establishing the
first metastatic colony on the omentum/peritoneum, acting as
cancer-associated mesothelial cells.

Methods

Further information can be found in Supplemental Methods.
Reagents. Collagen I (rat tail) and fibronectin were purchased from
BD Biosciences. The human OvCa cell lines, SKOV3ip1 and HeyA8, were
provided by G.B. Mills (M.D. Anderson Cancer Center, Houston, Tex-
as, USA). The mouse OvCa cell line ID8 was provided by K. Roby (Uni-
versity of Kansas Medical Center, Kansas City, Kansas, USA; ref. 50).

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The cell lines were validated by short tandem repeat DNA fingerprinting using the AmpFLSTR Identifier kit (Applied Biosystems) and compared with known American Type Culture Collection fingerprints, the Cell Line Integrated Molecular Authentication database (CLIMA), and the University of Texas MD Anderson Cancer Center fingerprint database. Negative control, fibroblast, in vivo fibronectin, and vitronectin siRNA were purchased from Ambion Inc.; SMAD3 and TGF-β1 siGENOME siRNA were purchased from Dharmacon. The TGF-βRII antibody (catalog no. 616451) (51), TGF-βRII kinase inhibitor II (catalog no. 616452) (52), and RHO kinase inhibitor (catalog no. 555552) (53) were from Calbiochem. The Ras signaling activity inhibitor (catalog no. 10010501) (54) was from Cayman Chemical. The RAC-GEF interaction inhibitor (catalog no. 2161) was from Tocris Bioscience (55). The TGF-βRII antibody (catalog no. AF-241-NA) was from R&D Systems (56). The β1 integrin (clone P5D2) antibody was purchased from Santa Cruz. The β1 integrin antibody (clone 339.1) was provided by Biozzi. The β1 integrin (OS2966) antibody was provided by Biozzi. The αV integrin (clone P1D6) antibodies were purchased from Biozzi. The αV integrin (clone 224) antibody was purchased from Cell Signaling Technology. Ad5CMVCre-eGFP and Ad5CMV-eGFP were purchased from University of Missouri.

TMA and immunohistochemistry. TMA cores (n = 2) of omental metastases from 108 patients with serous papillary OvCa were collected (Supplemental Table 3). The patients were selected for the TMAs by a gynecologic pathologist. Clinical and histopathologic information was collected and updated as previously reported (57). TMA slides were deparaffinized and incubated with anti-fibronectin (Sigma-Aldrich) at 1:400 dilution as previously described (25, 39). Slides were stained using the Envision avidin-biotin-free detection system and visualized with enhanced chemiluminescence reagents.

For coculture assays, full human omentum pieces were cultured with 1 × 10^6 fluorescently labeled SKOV3ip1 cells. After incubation at 37°C for 4 hours, omental pieces were washed 3 times in PBS, digested in 5% NP-40 for 30 minutes at 37°C, and scraped with a metal spatula. All cells removed during digestion were placed in a 24-well plate, and the total fluorescent intensity per well was quantified. Adhesion assays were run in quadruplicate.

For coculture assays, full human omentum pieces were cultured with 1 × 10^6 SKOV3ip1 cells. After incubation at 37°C for 48 hours, omental pieces were digested in 0.2N NH4OH. The remaining ECMs were washed 3 times with PBS, collected, and analyzed using SDS-PAGE. The protocol was adapted from a previously described method (60).

qRT-PCR. After coculture of GFP-labeled OvCa cells with full human omentum, the 3D omental culture, or primary human mesothelial cells, cells were sorted by FACS in PBS (39). This procedure separated labeled OvCa cells from mesothelial cells and/or fibroblasts after their coculture. TRIZOL reagent was used to isolate RNA according to the manufacturer’s instructions (Invitrogen). cDNA was synthesized using the Applied Biosystems cDNA archive kit. After reverse transcription, real-time PCR was performed using a Prism7500 TaqMan PCR detector (Applied Biosystems) with presigned and validated TaqMan probes for FNI, CDHI (encoding E-cadherin), and VIM (encoding vimentin) in conjunction with GAPDH for normalization (Applied Biosystems) (58). The reactions were run in triplicate. Relative levels of mRNA expression were calculated using the 2^-ΔΔCt method (61). Differences between treatments were evaluated using unpaired, 2-tailed Student’s t test.

Immunoblot analysis. For analysis of cellular fibronectin, tissue or cells after FACS were lysed, and equal quantities of protein were added to each blot. For analysis of secreted ECM, equal volumes of ECM were added to each blot. For analysis of tumor ECM, equal quantities of protein were added to each blot. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose, and immunoblot analysis was performed (58). The following antibodies were applied overnight at 4°C: anti-fibronectin (1:2,000 dilution); anti–phospho-SMAD2/3 (1:1,000), anti–E-cadherin (1:2,000), anti-vimentin (1:2,000), anti–GAPDH (1:2,000), mouse anti-actin (1:50,000). Blots were then incubated with secondary horseradish peroxidase–conjugated IgG and visualized with enhanced chemiluminescence reagents.

Ex vivo full human omentum culture. For omental cultures, a fresh piece of full human omentum was cut into 8-mm pieces (equivalent weights) (14). All omental pieces were placed in a 24-well dish (1 piece/well). For the adhesion assay, full human omentum pieces were transfected with in vivo fibronectin or control siRNA 24 hours prior to coculture with 1 × 10⁶ fluorescently labeled SKOV3ip1 cells. After incubation at 37°C for 4 hours, omental pieces were washed 3 times in PBS, digested in 5% NP-40 for 30 minutes at 37°C, and scraped with a metal spatula. All cells removed during digestion were placed in a 24-well plate, and the total fluorescent intensity per well was quantified. Adhesion assays were run in quadruplicate.

For coculture assays, full human omentum pieces were cultured with 1 × 10⁶ SKOV3ip1 cells. After incubation at 37°C for 48 hours, omental pieces were digested in 0.2% trypsin-EDTA for 5 minutes at 37°C and scraped with a metal spatula. The trypsin was neutralized, and collected cells were pelleted. For analysis of fibronectin expression and production, GFP-labeled SKOV3ip1 cells and omental surface cells were sorted by FACS.

Primary omental cells and 3D omental culture. Primary human mesothelial cells and fibroblasts were isolated from the omentum, and purification was verified by vimentin, keratin 8, and prolyl-hydroxylase
immunohistochemistry (25, 39). The 3D omental culture system was assembled by plating 4,000 human primary fibroblasts per well plus 0.5 μg collagen type I. 4 hours later, human primary mesothelial cells were added at a density of 20,000 cells in 0.33 cm².

**Adhesion assay.** Omental ECM (0.5 μg), tumor ECM (0.5 μg), or 20,000 primary human mesothelial cells were used to coat a 96-well dish (0.33 cm²), and a 1-hour adhesion assay was performed with 40,000 fluorescently labeled OvCa cells (39). Cells were treated with mouse IgG, β1 integrin, αv integrin, αβ3 integrin, or β3 integrin antibodies (20 μg/ml) at the time of cell plating. Differences between treatments were evaluated using unpaired, 2-tailed Student’s t test.

** Invasion assay.** 50,000 fluorescently labeled OvCa cells were plated on each well of a 24-well transwell plate (0.33 cm²) that was precoated with 20 μg omental ECM, precoated with 20 μg tumor ECM, or contained 20 μg collagen type I with 20,000 primary human mesothelial cells. Cells were treated with mouse IgG, β1 integrin, αv integrin, αβ3 integrin, or β3 integrin antibodies (20 μg/ml) at the time of cell plating. The invasion assays were performed as previously described (39). Differences between treatments were evaluated using unpaired, 2-tailed Student’s t test.

**IF.** GFP-labeled SKOV3ip1 cells were added to a culture of primary human mesothelial cells or fibronectin-coated (100 μg) glass coverslips for 48 hours (25). The cells were fixed with 4% paraformaldehyde in PBS for 30 minutes, and IF was performed. Primary antibodies against fibronectin (1:400 dilution) and secondary antibodies (goat anti-rabbit Alexa Fluor 645, 1:200 dilution; Invitrogen) were used. Intracellular fibronectin was analyzed using Triton-X detergent in buffers (0.1%), while extracellular fibronectin was analyzed using buffers lacking detergents. The Triton-X detergent permeabilizes the cell membrane, allowing for intracellular fibronectin detection while washing away extracellular fibronectin. Nuclear counterstain was performed with Hoechst. Imaging was performed using a Zeiss LSM510 confocal microscope.

**DOC solubility assay.** The assay was based on the insolubility of fibronectin matrix in 2% DOC detergent (62). Cell pellets were lysed in DOC lysis buffer (2% sodium DOC, 20 mM Tris-HCl pH 8.8, 2 mM PMSF, 2 mM EDTA, 2 mM iodoacetic acid, 2 mM N-ethylmaleimide). Lysates were centrifuged to separate DOC-insoluble matrix from DOC-soluble material containing cell-associated and intracellular fibronectin. The DOC-insoluble fibronectin was solubilized in a buffer containing 1% SDS (20 mM Tris-HCl pH 8.8, 2 mM PMSF). The DOC-soluble and -insoluble fractions were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting.

**Inhibition experiments.** Human OvCa cell lines SKOV3ip1 and HeyA8 were added to a confluent culture of primary human mesothelial cells and treated with RAC inhibitor (5 μM), RAS inhibitor (25 μM), RHO inhibitor (10 μM), TGF-βRII inhibitors (10 μM), or anti-TGF-βRII (8 μg/ml) neutralizing antibody. Fibronectin production and secretion was analyzed 48 and 72 hours later, respectively. Fibronectin, SMAD3, TGF-βRII, and control siRNA constructs were transfected into 2 × 10⁴ primary human mesothelial cells 18 hours prior to coculture with SKOV3ip1 cells. SKOV3ip1 cells were transfected with TGF-β1 or control siRNA constructs using Lipofectamine Transfection Reagent (Invitrogen). The cells recovered in full growth media (10% fetal bovine serum) for 18 hours, and serum-free media was added. SKOV3ip1 cell CM was collected after 48 hours and concentrated (10×) using a spin column with 3-μm filter (Millipore).

**RAC activity measurement.** Immunoprecipitation-immunoblot was performed as described by the manufacturer (Cell Biolabs Inc.). Briefly, 1 × 10⁶ SKOV3ip1 cells were cultured alone or added to a culture of 4 × 10⁶ primary human mesothelial cells for 24 hours. Cells were washed with PBS, lysed, scraped, collected, and pelleted. The supernatant was incubated with 0.05M EDTA for 30 minutes at 30°C. GTPyS or GDP were added to duplicate samples for positive and negative controls, and loading was quenched with 0.2M MgCl₂, treatment. RAC1 pulldown assay was performed. Cell lysates were incubated with PAK PDB agarose beads and incubated at 4°C for 1 hour. The beads were pelleted, washed, and boiled in 2× reducing SDS-PAGE sample buffer. The supernatant was resolved by SDS-PAGE and transferred to nitrocellulose, and immunoblot was performed. Anti-RAC1 antibody (1:1,000 dilution) was applied overnight at 4°C. Blots were then incubated with secondary horseradish peroxidase-conjugated IgG and visualized with enhanced chemiluminescence reagents.

**Transfections.** Primary human mesothelial cells were transiently transfected with the full-length FN1 promoter (-1,200 bp) (28) using Lipofectamine Transfection Reagent (Invitrogen) (39). At 18 hours after transfection, SKOV3ip1 cells (1.5 × 10⁶/well) were trypsinized and added on the transfected mesothelial cells. After 12 hours, cells were detached and lysed, and luciferase was analyzed (63).

**In vivo mouse OvCa coculture.** Athymic nude mice received i.p. injection of 4 × 10⁶ HeyA8 cells (39). 48 hours after injection, the mouse omentum was removed, digested (0.2% trypsin-EDTA) for 5 minutes at 37°C, and scaped with a metal spatula. The trypsin was neutralized, collected cells were pelleted, and fibronectin production was analyzed.

**Fn1fl/fl mouse experiments.** Fn1fl/fl mice — generated by R. Fassler (Max-Planck Institute of Biochemistry, Martinsried, Germany) and provided by S. Dallas (University of Missouri, Kansas City, Missouri, USA) — have been previously described (29, 64). The mice were bred in house and regularly genotyped (29). Fn1fl/fl mice have no reported or observed abnormalities, are fertile, and have a normal lifespan.

In order to generate mice with tissue-specific deletion of Fn1, Cre must be expressed in a tissue-specific and/or inducible manner (29).

Therefore, Fn1fl/fl mice received i.p. injections of Ad-Cre (Ad5CMVCre) to delete fibronectin in the lining of the peritoneal cavity, or i.p. injections of Ad-Cont (Ad5CMV), 96 hours prior to cancer cell injection. 4 × 10⁶ GFP-labeled ID8 mouse OvCa cells (65) were injected; 72 hours later, the mouse omentum was removed and imaged by fluorescent microscopy. ID8 and omental surface cells were digested (0.1% NP-40) off of the omentum, and total fluorescence of bound cells was measured using a fluorescent plate reader (BioRad). Adenovirus infection (GFP-labeled cells) was confirmed in peritoneal surface cells, and allele-specific PCR validated the recombination of floxed Fn1 in peritoneal surface cells 48 hours after i.p. adenovirus injection. To test the effect of an α5 integrin blocking antibody in addition to fibronectin knockdown, 4 × 10⁶ GFP-labeled ID8 cells were preincubated for 15 minutes with 20 μg/ml α5 integrin antibody (clone 339.1) or mouse IgG before i.p. injection.

Peritoneal surface cells from Fn1fl/fl mice were isolated, cultured, and infected with Ad-Cre or Ad-Cont. Adhesion, invasion, and proliferation of ID8 cells were examined on the adenovirus-infected peritoneal surface cells, as described above. The adenovirus infection was efficient (>92%; infection at MOI 50), as observed with EGFP expression, and did not reduce viability (MOI <100). Recombination of floxed Fn1 was verified by allele-specific PCR (see below).
Treatment studies. The effect of α5 integrin (66) or β3 integrin (38) antibody on OvCa cell adhesion, invasion, and proliferation in vitro was investigated. Mouse IgG (control) or α5 integrin or β3 integrin antibody (10 μg/ml) was added to fluorescently labeled SKOV3ip1 and HeyA8 OvCa cells 15 minutes prior to performing assay and adhesion (2 hours), invasion (24 hours), or proliferation (96 hours) assays on the preplated 3D omental culture (25).

For the in vivo study, 2.5 × 10⁶ HeyA8 cells were mixed with mouse IgG (control) or α5 integrin or β3 integrin antibody (10 μg/ml), and 10 μl was injected into the ovaries of athymic nude mice. At 2 and 4 days after injection, mouse IgG or α5 integrin or β3 integrin antibody in PBS (10 mg/kg/d) was administered i.p. At 28 days after injection, mice were sacrificed (10 per group). The number of tumor colonies was counted, and the tumor was weighed (57).

Statistics. Adhesion (n = 5) and invasion (n = 3) assays were performed and at least 3 independent experiments conducted. Mean and SEM are reported. Significance of differences was determined by 2-tailed independent (unpaired) t test; a P value less than 0.05 was considered significant.

Study approval. Specimens of human omentum were obtained from patients undergoing surgery for benign conditions, and tumor samples from OvCa patients undergoing surgery, at the University of Chicago. All human specimens were obtained in accordance with the protocol approved by the University of Chicago IRB, and subjects provided informed consent. All animal procedures were approved by the University of Chicago’s policies on the care, welfare, and treatment of laboratory animals.

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