Heterotypic CAF-tumor spheroids promote early peritoneal metastasis of ovarian cancer

Qinglei Gao1*, Zongyuan Yang1*, Sen Xu1, Xiaoting Li1, Xin Yang1, Ping Jin1, Yi Liu1, Xiaoshui Zhou1, Taoran Zhang1, Cheng Gong1, Xia Wei1, Dan Liu2, Chaoyang Sun1, Gang Chen1, Junbo Hu1, Li Meng2, Jianfeng Zhou2, Kenjiro Sawada3, Robert Fruscio4,5, Thomas W. Grunt6, Jörg Wischhusen6, Víctor Manuel Vargas-Hernández8, Bhavana Pothuri9, and Robert L. Coleman10

High-grade serous ovarian cancer (HGSOC) is hallmarked by early onset of peritoneal dissemination, which distinguishes it from low-grade serous ovarian cancer (LGSOC). Here, we describe the aggressive nature of HGSOC ascitic tumor cells (ATCs) characterized by integrin α5high (ITGA5high) ATCs, which are prone to forming heterotypic spheroids with fibroblasts. We term these aggregates as metastatic units (MUs) in HGSOC for their advantageous metastatic capacity and active involvement in early peritoneal dissemination. Intriguingly, fibroblasts inside MUs support ATC survival and guide their peritoneal invasion before becoming essential components of the tumor stroma in newly formed metastases. Cancer-associated fibroblasts (CAFs) recruit ITGA5high ATCs to form MUs, which further sustain ATC ITGA5 expression by EGF secretion. Notably, LGSOC is largely devoid of CAFs and the resultant MUs, which might explain its metastatic delay. These findings identify a specialized MU architecture that amplifies the tumor-stroma interaction and promotes transcoelomic metastasis in HGSOC, providing the basis for stromal fibroblast-oriented interventions in hampering OC peritoneal propagation.

Introduction

High-grade serous ovarian cancer (HGSOC)—the most aggressive form of ovarian cancer (OC)—is characterized by insidious onset, rapid i.p. spread, and the development of massive ascites (Vaughan et al., 2011; Konecny et al., 2014; Nik et al., 2014). However, its low-grade serous ovarian cancer (LGSOC) counterpart progresses slowly and has a more favorable outcome (Schneler and Gershenson, 2008; Imamura et al., 2015). Although their distinct molecular origins have been elucidated, the mechanisms mediating this discrepant biology relative to peritoneal spreading are poorly understood (Tung et al., 2009; Angarita et al., 2015). In any case, to establish peritoneal metastases, tumor cells must escape from the primary tumor site as either single cells or spheroids (Wintzell et al., 2012; Auer et al., 2017), adhere to the mesothelial layer covering the abdominal cavity, and subsequently invade the favored extracellular matrix (ECM)—rich compartment (Kenny et al., 2011, 2015). Previous studies had emphasized that ascitic spheroids represent the invasive and chemoresistant cellular population fundamental to metastatic dissemination (Barbone et al., 2008; Sodek et al., 2009; Lawrenson et al., 2011). Little attention, however, has been devoted to analyzing tumor spheroid composition and ascitic tumor cell (ATC) heterogeneity in HGSOC patients and even less so in LGSOC patients. Considering the crucial role of peritoneal adhesion and the proposed function of spheroids during OC metastasis, we sought to investigate the processes by which ATCs assemble to form ascitic spheroids and subsequently execute peritoneal dissemination.

Ascites represents a tumor microenvironment that is rich in various cellular elements, cytokines, and ECM components (Ahmed and Stenvers, 2013; Thibault et al., 2014; Chudecka-Glaz et al., 2015). Development of ascites typically occurs upon dissemination of tumor cells into the peritoneum, before implantation of solid metastases. Ascites development is associated with disease progression in HGSOC patients (Kipps et al., 2013). Constant interactions between tumor cells and other components within the ascites fluid could significantly shape the malignant phenotype. For instance, ascites sustains a high...
percentage of cancer stem cells that contribute to disease recurrence and chemoresistance (Bapat et al., 2005; Latifi et al., 2012). Biomechanical factors such as fluidic force perturbations can also contribute to further tumor cell dissemination and metastatic progression (Rizvi et al., 2013). Importantly, the harsh hypoxic and anoikis-prone ascitic environment exerts a strong selective pressure on ATCs, thus allowing only the fittest cells to survive. Recently, tumor-associated macrophages (TAMs) were shown to drive spheroid formation and transcoelomic metastasis (Yin et al., 2016). Such mechanistic insights indicate that the ascites microenvironment can serve as a valuable platform to characterize ATCs with the intention of developing more effective therapies.

Therefore, we set out to investigate the intrinsic heterogeneity of ATCs and their contribution to OC progression. Our present study for the first time describes a critical role of cancer-associated fibroblast (CAF)–centered heterotypic spheroids, which represent metastatic units (MUs) in OC peritoneal adhesion and metastasis. The stromal fibroblast backbone recruits detached ATCs to form MUs at early stages of transcoelomic metastasis. We uncovered that integrin α5 (ITGA5) is indispensable for ATCs in forming MUs with CAFs. Moreover, epidermal growth factor (EGF) derived from activated fibroblasts inside the compact MU microenvironment further sustains ATC ITGA5 expression, which strengthens tumor–stromal interaction inside MUs. Our results thus imply that different interactions with ascitic CAFs and the resultant MU architecture might underlie the distinct patterns of peritoneal metastasis in HGSOC and LGSOC.

Results
HGSOC ATCs display an aggressive nature
To investigate the metastatic potential of ascites-derived tumor cells (ATCs), we isolated tumor epithelial cells from matched primary tumors, ascites, and solid metastases of HGSOC patients (Fig. 1A). In vitro and in vivo adhesion assays both revealed that ATCs adhered more rapidly and securely to ECM substrate than matched primary and metastatic tumor cells (Fig. 1, B and C). Further analysis showed that ATCs were more invasive and exhibited enhanced mesothelial clearance capacity (Fig. 1, B and D), and mesothelial cell invasion (Fig. 1, A and B). Importantly, the harsh hypoxic and anoikis-prone ascitic environment exerts a strong selective pressure on ATCs, thus allowing only the fittest cells to survive. Recently, tumor-associated macrophages (TAMs) were shown to drive spheroid formation and transcoelomic metastasis (Yin et al., 2016). Such mechanistic insights indicate that the ascites microenvironment can serve as a valuable platform to characterize ATCs with the intention of developing more effective therapies.

Therefore, we set out to investigate the intrinsic heterogeneity of ATCs and their contribution to OC progression. Our present study for the first time describes a critical role of cancer-associated fibroblast (CAF)–centered heterotypic spheroids, which represent metastatic units (MUs) in OC peritoneal adhesion and metastasis. The stromal fibroblast backbone recruits detached ATCs to form MUs at early stages of transcoelomic metastasis. We uncovered that integrin α5 (ITGA5) is indispensable for ATCs in forming MUs with CAFs. Moreover, epidermal growth factor (EGF) derived from activated fibroblasts inside the compact MU microenvironment further sustains ATC ITGA5 expression, which strengthens tumor–stromal interaction inside MUs. Our results thus imply that different interactions with ascitic CAFs and the resultant MU architecture might underlie the distinct patterns of peritoneal metastasis in HGSOC and LGSOC.

Results
HGSOC ATCs display an aggressive nature
To investigate the metastatic potential of ascites-derived tumor cells (ATCs), we isolated tumor epithelial cells from matched primary tumors, ascites, and solid metastases of HGSOC patients (Fig. 1A). In vitro and in vivo adhesion assays both revealed that ATCs adhered more rapidly and securely to ECM substrate than matched primary and metastatic tumor cells (Fig. 1, B and C). Further analysis showed that ATCs were more invasive and exhibited enhanced mesothelial clearance capacity (Fig. 1, B and D), and mesothelial cell invasion (Fig. 1, A and B). Importantly, the harsh hypoxic and anoikis-prone ascitic environment exerts a strong selective pressure on ATCs, thus allowing only the fittest cells to survive. Recently, tumor-associated macrophages (TAMs) were shown to drive spheroid formation and transcoelomic metastasis (Yin et al., 2016). Such mechanistic insights indicate that the ascites microenvironment can serve as a valuable platform to characterize ATCs with the intention of developing more effective therapies.

Therefore, we set out to investigate the intrinsic heterogeneity of ATCs and their contribution to OC progression. Our present study for the first time describes a critical role of cancer-associated fibroblast (CAF)–centered heterotypic spheroids, which represent metastatic units (MUs) in OC peritoneal adhesion and metastasis. The stromal fibroblast backbone recruits detached ATCs to form MUs at early stages of transcoelomic metastasis. We uncovered that integrin α5 (ITGA5) is indispensable for ATCs in forming MUs with CAFs. Moreover, epidermal growth factor (EGF) derived from activated fibroblasts inside the compact MU microenvironment further sustains ATC ITGA5 expression, which strengthens tumor–stromal interaction inside MUs. Our results thus imply that different interactions with ascitic CAFs and the resultant MU architecture might underlie the distinct patterns of peritoneal metastasis in HGSOC and LGSOC.
lin and developed robust FAs (Fig. 1 F). These cells also formed larger patient-derived xenograft tumors and more micrometas-
tases compared with their counterparts from primary tumor and
metastases (Fig. 1, G and H). In the context of LGSOC—an OC
subtype that is clinically and molecularly distinct from HGSOC
(Diaz-Padilla et al., 2012)—parallel experiments were performed. These revealed that ATCs displayed nearly equal capacity with re-
spect to adhesion, invasion, and mesothelial clearance, compared
with their matched counterparts (Fig. S1, A–D).

**ITGA5* ATCs are fundamental to OC peritoneal metastasis**

The distinct characteristics of ATCs drove us to further explore their specific phenotype. After isolation of matched tumor epithe-

telial cells from primary, ascites, and omentum metastatic loci

from HGSOC patients, the transcriptome of ATCs clearly differed

from primary and metastatic tumor cells, which were quite similar
to each other (Fig. 2 A). The comparison between ATCs and
either primary or metastatic tumor cells revealed 1,138 and 1,162
genes, respectively, to be overexpressed twofold or more in ATCs
(Tables S1 and S2), whereas only 21 genes were upregulated two-
fold or more between metastatic and primary tumor cells (Table
S3). The overlapping set of 712 genes overexpressed in the ATCs
was enriched for genes involved in the immune response, cell
adhesion, and integrin-mediated signaling (Fig. 2 B). A similar comparison with cells derived from patients harboring HGSOC
revealed that cells in the primary tumor showed a gene expres-
sion pattern which was distinct from that of ascites and meta-
static tumor cells, whereas the transcriptional profile of ATCs
was highly comparable to that of metastatic tumor cells. The most
pronounced genetic variation in LGSOC was observed between
metastatic and primary tumor cells, in which the up-regulated
gene sets were enriched for genes involved in cell cycle regula-
tion, cell adhesion, and multicellular organismal development.
No significant functional enrichment was noted in the genes ele-
vated in ATCs compared with either primary or metastatic tumor
cells (Fig. S1, E and F). As previous studies highlighted the pivotal
role of the integrin family in mediating OC peritoneal adhesion
and metastasis (Casey and Skubitz, 2000; Elmasri et al., 2009),
we further analyzed the expression of the over-represented in-
tegrins and observed that ITGA5 and β3 were specifically up-reg-
ulated in HGSOC ATCs (Fig. 2 C), whereas no specific dominant
integrin expression was noted in LGSOC ATCs (Fig. S1 G). ITGA5
was selected for further analysis due to its recognized role in ad-
hesion regulation and correlation with worse patient outcome in
OC (Sawada et al., 2008; Mitra et al., 2011).

Enhanced expression of ITGAS and vimentin (Vim) and re-
duced CDH1 (E-cadherin) expression were confirmed by immu-
noblotting in matched tumor epithelial cells (Fig. 2 D). The inverse correlation between ITGA5 and the epithelial marker

CDH1 was further validated in NCI-60 cells (R = −0.5965, P <
0.0001) and The Cancer Genome Atlas (TCGA) datasets (R =
−0.1976, P < 0.0001; Fig 2 E), indicating that ITGA5* cells rep-
resent a mesenchymal OC subpopulation. Both ITGA5 mRNA and
protein levels correlated with worse patient outcome in public
databases and in our panel of HGSOC specimens (Fig. 2, F and G).
To assess the effects of loss of ITGA5 on tumor growth in vivo, we
used CRISPR/Cas9 technology to knock down ITGA5 in SKOV3
cells. Three single guide RNAs (sgRNAs) were designed, and ablation of ITGA5 was most evident in tumor cells edited with
sgRNA2, confirmed with T7 endonuclease I (T7EI) assay and im-
munoblotting (Fig. 2, H and I). Subsequently, ITGA5-deficient
(cells (sgRNA2 group) observed to form smaller aggregates
and displayed a markedly decreased capacity for adhesion com-
pared with control HGSOC cell line OV90 and the ascites-derived
ovarian adenocarcinoma cell line SKOV3 (Fig. 2 J). In addition, an
in vivo assay further revealed that the majority of cells adhering
to the metastatic “tropism” omentum and mesentery were PKH-
67–labeled ITGA5 complete cells (Fig. 2 K). Finally, biolumines-
cence imaging revealed that loss of ITGA5 inhibited growth of
peritoneal OC xenografts over time (Fig. 2, L and M). Collectively,
these data suggest that the ITGA5* cells in the ascites are cru-

tial for peritoneal adhesion and metastasis.

**ATCs form heterotypic spheroids, named MUs, with CAFs in
HGSOC and discriminate from LGSOC**

The phenotype and biology of ATCs is shaped by influences from
ascites that constitutes their unique resident microenvironment.
Malignant ascites is not only a common feature of advanced OC,
but it also nourishes and constantly modulates the behavior of
disseminated tumor cells which adapt to their new environment
to become resident ATCs (Penson et al., 2000; Lane et al., 2011).
In the context of ascites fluid collected from HGSOC and LGSOC
patients, we observed that the HGSOC ascitic cells combine to form
spheroids, whereas the LGSOC ascitic cells are prone to exist in
solitary form or as smaller aggregates (Fig. 3 A). Besides tumor
epithelial cells (EpCAM*; epithelial cell adhesion molecule), CAFs
(FAP*; fibroblast activation protein), immune cells (CD45*), and
endothelial cells (CD31*) are the main cell types in ascites (Erez
et al., 2010). A subsequent compositional analysis identified fi-
broblasts (EpCAM* CD45* CD31* FAP*) as the cellular component
showing the largest difference between HGSOC and LGSOC ascites
(Fig. 3, B and C). These observations raise the possibility that asc-
itic fibroblasts might influence ATC behavior and OC progression.
Indeed, immunofluorescence analysis revealed that HGSOC-
derived ascitic spheroids contained architectures characterized
by EpCAM* epithelial cells surrounding a core of CAFs, charac-
terized by α-smooth muscle actin (α-SMA), platelet-derived
growth factor receptor-β (PDGFRB), or prolyl 4-hydroxylase
staining (Fig. 3 D). Therefore, we hypothesized that CAFs partic-
ate in ascitic spheroid formation and subsequent transcoelo-
omic metastasis of OC. We established a suspension coculture of
SKOV3 and CAFs and found that tumor cells were present in the
form of single cells, SKOV3-only spheroids (homospheroids), and
SKOV3/CAF heterotypic spheroids (heterospheroids). Indeed,
heterospheroids displayed the strongest adhesive capacity, fol-
lowed by homospheroids, and then single tumor cells (Fig. S2, A
and B). Subsequent anoikis assays revealed that tumor cells in the
heterospheroids displayed the lowest apoptosis rate in suspen-
sion culture (Fig. S2 C), further supporting that spheroid tumor

cells are more resistant and prone to metastasis. Moreover, het-

erospheroids show enhanced mesothelial clearance, invasion,
and spreading capabilities (Fig. S2, D–F).

To further explore the contribution of CAFs and associated
heterospheroids during early OC transcoelomic metastasis, we

Gao et al.
Metastatic unit in high-grade serous OC

Journal of Experimental Medicine

https://doi.org/10.1084/jem.20180765
established a mouse model in which PKH-26–labeled CAFs and GFP-transfected SKOV3 were i.p. injected into NOD-SCID female recipient mice (Fig. 3 E). Intriguingly, typical heterospheroids formed instantaneously and adhered to their metastatic tropism omentum (Fig. 3, F and G), followed by spreading of heterospheroids and subsequent development of pronounced protruding tumor nodules with a CAF-derived stroma (Fig. 3, H and I). Consequently, characteristic heterospheroids appeared in murine ascites that had developed at the end of the experiment (Fig. 3 J). We conclude that exogenous CAFs support adhesion and metastasis of tumor cells by forming heterospheroids.

To further demonstrate the involvement of endogenous CAFs in mediating heterotypic spheroid formation and early transcoelomic metastasis, GFP-transfected SKOV3 cells were implanted either i.p. or orthotopically. Heterospheroids could also be observed early after implantation in the murine peritoneal cavity, with GFP+ tumor cells surrounding the GFP− stromal cells. Subsequent constitutional analyses revealed that fibroblasts (EpCAM CD45−CD31+ FAP+) were the major ingredient of GFP− host stromal cells (Fig. S3, A–D). This phenomenon was further observed in the EGFP transgenic mice after peritoneal injection of PKH-26–labeled syngeneic ID8 tumor cells, which resulted in the development of ascitic spheroids consisting of GFP+ stromal cells surrounded by tumor cells. Again, fibroblasts proved to be the major component of the GFP+ core population by flow cytometry analyses and immunofluorescence (Fig. S3, E–G). Due to its inherent malignant potential and contribution to peritoneal dissemination, we termed this CAF-centered, compact, heterospheroid structure an MU.
HGSOC derived CAFs facilitates MU formation and peritoneal metastasis of LGSOC ATCs

Our previous results had already shown that CAFs are rare in the peritoneal cavity of LGSOC patients, which was further validated by immunoblotting of representative fibroblast activation markers in ascitic cells from HGSOC and LGSOC patients (Fig. 4 A). To evaluate whether exogenous CAFs could alter the biological behavior of LGSOC ATCs, we compared the adhesion and metastatic capacity of them in the absence or presence of HGSOC-derived CAFs. Intriguingly, addition of HGSOC-derived CAFs to ATCs from LGSOC facilitated formation of typical heterotypic spheroids and subsequent peritoneal adhesion (Fig. 4, B and C). Consequently, ATCs derived from LGSOC showed notably enhanced tumor xenograft growth when HGSOC-derived CAFs were present (Fig. 4 D). Thus, the clinical observation of a slower disease progression natural history in LGSOC might be ascribed, in part, to the lack of ascitic CAFs and subsequent MUs.

ITGA5 mediates the adhesion of ATCs with CAFs in the formation of MUs

We reasoned that adhesion of tumor cells to CAFs is required for formation of MUs. As integrin family members are known to mediate tumor cell adhesion in spheroids (Leroy-Dudal et al., 2005; Suzuki et al., 2005; Santiago-Medina and Yang, 2016), we compared the expression pattern of tumor cells that could form MUs with CAFs and those remaining individual tumor cells. SKOV3 in spheroids and individual SKOV3 cells were thus isolated and subjected to molecular profiling (Fig. 5 A). Significantly, ITGA5 was identified as the second-most elevated gene in spheroid-SKOV3 compared with individual SKOV3 cells.
Among the integrin family members, integrin α2, α5 and β4 were significantly higher in spheroid-SKOV3 compared with individual SKOV3 cells (Fig. 5 C). To specifically assess the involvement of the aforementioned integrins in MU formation, we employed specific neutralizing antibodies to block them and found that blocking of ITGA5 could drastically blunt MU generation by CAFs with SKOV3 and OV90 cells (Fig. 5, D and E).

In addition, tumor cells exhibited elevated ITGA5 and VIM levels and reduced integrin β1 (ITGB1) and CDH1 levels after suspended coculture with CAFs in MUs, thus indicating that interaction with CAFs in MUs could further maintain the ITGA5high
mesenchymal phenotype in ATCs (Fig. 5F). Immunoblotting in several primary ATCs further revealed that ITGA5 was notably up-regulated after coculture in MUs with CAFs (Fig. 5G). In the context of OC tumor tissues, ITGA5 staining was predominately detected at the invasive front of HGSOC specimens and areas adjacent to stromal CAFs (Fig. 5, H and I). These data indicate that the fibroblast backbone may selectively attract ITGA5high ATCs to form MUs, in which ITGA5 expression by ATCs is then maintained.

**EGF derived from CAFs within MUs is required to maintain ITGA5 expression in ATCs**

Numerous studies have revealed that interactions between tumor and stroma depend on cytokines (Orimo et al., 2005; Chen et al., 2014). To identify the cytokines secreted by activated fibroblasts that sustain ITGA5 expression in ATCs, we evaluated the cytokine profiles of conditioned medium (CM) from control CAFs and from CAFs activated by ITGA5high SKOV3–derived CM or by transforming growth factor β (TGF-β1), which was introduced as an inducer or positive control of fibroblast activation. This revealed nine cytokines (EGF, IP-10, IGF-BP-3, BDNF, Flt-3 LG, FGF-7, IL-12, MIF, and leptin) that were up-regulated in both groups (Fig. 6A and Table S5). Among them, EGF displayed the highest correlation with ITGA5 in SKOV3 tumor cells (Fig. 6B). Moreover, EGF expression in CAFs increased following addition of OC cell CM or TGF-β1 and was suppressed in the presence of TGF-β1–neutralizing antibody (Fig. 6A, A and B). Interestingly, immunofluorescence revealed amplified EGF expression in heterospheroids, which was not readily apparent in homospheroids, solitary tumor cells, or CAFs (Fig. 6C). Accordingly, EGF was much more prevalent in the MU microenvironment compared with the corresponding ascites macroenvironment of HGSOC patients (Fig. 6D). The importance of EGF for increased ITGA5 expression in tumor cells was confirmed, since addition of an EGF-neutralizing antibody to the suspension coculture attenuated ITGA5 expression in MUs (Fig. 6E). Reporter assays also demonstrated that ITGA5 promoter activity was inhibited tremendously in the presence of EGF-neutralizing antibody in spheroid coculture (Fig. 6F).

Immunostaining in a series of HGSOC tissues revealed that EGF was evenly distributed between epithelial and stromal regions, whereas EGFR was predominantly located in epithelial cells (Fig. S4, C and D). Quantitative PCR analysis in paired tumor cells and CAFs confirmed the above expression pattern of EGF and EGFR (Fig. S4E), suggesting that EGF in OC microenvironment mainly acts on cancer cells where it helps to maintain
their ITGAS\textsuperscript{high} phenotype. Accordingly, neutralizing anti-EGF antibody blunted the formation of MUs by SKOV3 and CAFs (Fig. 6 G). Finally, the EGF neutralizing antibody attenuated peritoneal tumor burden and increased survival in mice following peritoneal injection of SKOV3-Luc cells and CAFs (Fig. 6, H and I), accompanied by diminished ITGAS expression in ATCs and reduced metastases in the anti-EGF treatment group compared with the IgG group (Fig. 6 J). To further ascertain the role of EGF/EGFR signaling in OC peritoneal dissemination, we used CRISPR/Cas9 editing to knock down EGFR in OC tumor cells. Ablation of EGFR was most evident in tumor cells edited with sgRNA3, confirmed with T7E1 assay and Western blot (Fig. 6, K and L). Tumor growth curves indicated that EGFR-deficient tumor cells showed notably diminished growth rate than EGFR complete SKOV3-Luc coinjected with CAFs (Fig. 6 M). These data suggest that EGF derived from activated fibroblasts are amplified inside MUs and promote spheroid formation and ITGAS expression in attached tumor cells.

**Disruption of MU integrity by targeting CAFs attenuated early-stage peritoneal metastasis**

Experiments performed so far highlighted that the CAF backbone inside a MU recruits and carries ATCs to remote metastatic sites in HGSO patients, raising the possibility that therapeutic interventions targeting CAFs could disrupt MU integrity and thus hamper metastatic implantation in the peritoneum and beyond. Imatinib—a small-molecule tyrosine kinase inhibitor with activity against BCR-ABL, c-KIT, and PDGFR—was selected on the basis that PDGF signaling is important for CAF survival (Murata et al., 2011). Enhanced PDGFR expression in stromal fibroblasts (Fig. S5 A) provided a further rationale. At concentrations <20 nM, imatinib selectively suppressed OC primary CAF viability and had a negligible effect on OC cell, macrophage, and endothelial cell survival (Fig. 7 A and Fig. S5 B). As expected, the addition of imatinib completely destroyed the central CAF skeleton and thus prevented subsequent heterospheroid formation by CAFs and OC cells (Fig. 7 B). In addition, imatinib increased the apoptosis rate of GFP+ tumor cell in coculture with CAFs in MUs (Fig. 7 C). Moreover, basal EGF secretion by CAFs and in the CAF/tumor cell coculture system was dose-dependently decreased by imatinib (Fig. 7 D). Up-regulation of ITGAS in tumor cells after coculture with CAFs in heterospheroids was thus attenuated in the presence of imatinib (Fig. 7 E).

With imatinib’s blunting of CAF activity and their interaction with tumor cells, we observed a sharp reduction in peritoneal adhesion of MUs when CAFs were treated with imatinib before interacting with SKOV3 cells (Fig. 7 F). Finally, pretreatment with imatinib significantly reduced the role of exogenous CAFs in facilitating peritoneal metastasis and improved survival of mice bearing SKOV3-Luc peritoneal xenografts (Fig. 7, G–I). To further ascertain the ability of imatinib to interfere with tumor-promoting effects of endogenous activated fibroblasts in OC xenografts, we developed an orthotopic mouse model to explore different schemes of imatinib administration (Fig. S5 C). This revealed that only early-stage imatinib intervention was sufficient to decrease peritoneal tumor burden and improve mouse survival. Primary tumor growth, however, remained unaffected by imatinib in the SKOV3-Luc orthotopic model (Fig. S5, D–F). Both H&E and Masson trichrome staining revealed that tumors arising from the imatinib-pretreated and early-phase imatinib intervention groups displayed a less stroma-rich architecture (Fig. 7 J and Fig. S5 G).

Moreover, host immune cells, especially TAMs, were reported to interact with CAFs and drive spheroid formation in OC (Yin et al., 2016), which was supported by our finding that CD45+ immune cells appeared together with MUs developed in xenograft models. Therefore, we extended our research to evaluate depletion of TAM in ascitic microenvironment influence of OC dissemination, using liposome clodronate (LC) alone or in combination with imatinib in a BALB/c nude mouse xenograft model. Removal of macrophages via LC significantly retarded peritoneal tumor metastasis, reinforced the tumor-suppression role of imatinib, and thus improved mouse survival bearing orthotopic OC xenograft (Fig. S5, H–J). The above findings emphasized the non-negligible contribution of TAM in OC peritoneal dissemination besides CAFs. Collectively, these results demonstrate that targeting CAFs could disrupt metastasis-prone MUs, thus interfering with CAF-dependent tumor cell adhesion and OC dissemination.

**Discussion**

A detailed understanding of how ATCs contribute to further metastasis is crucial to better comprehend the biological complexity of HGSO. In this study, we defined an OC MU characterized by a CAF skeleton surrounded by tumor cells, which was prevalent in HGSO ascites and actively involved in peritoneal dissemination. ITGAS\textsuperscript{high} ATCs were selectively recruited by CAFs to form the unique heterotypic spheroids. CAFs thus support ATC survival, guide their further peritoneal and transperitoneal adhesion and invasion, and finally constitute the tumor stroma in newly formed metastases. Moreover, EGF derived from fibroblasts under ATCs stimulation was significantly enriched within MUs, where it increases ITGAS expression in ATCs, thereby further strengthening interactions between ATCs and CAFs (Fig. 8). Targeting CAFs could destroy MU integrity and limit peritoneal tumor cell implantation in an OC xenograft model. Thus, we provide mechanistic and clinical insight into the role of spheroid-associated CAFs in human OC progression.

The supporting role of stromal elements during nidation and expansion of metastatic colonies has been well recognized. Interestingly, a novel powerful mechanism including paracrine lipid supply of tumor cells from omental stromal cells has recently been described (Nieman et al., 2011). Here, we further expand the prometastatic function of the stroma to the very early steps of metastatic spread. Given its substantial population of malignant ATCs, as well as the presence of various recruited and transformed host cells, malignant ascites in OC should not merely be seen as a fluidic environment but rather as dispersed tumor tissue. Interestingly, stromal fibroblasts were abundant in ascites and are prone to associate with ATCs to form prometastatic MUs in HGSO. The aggressive form of heterotypic spheroids identified in our experiments differs from previously reported multicellular aggregates in ascites by comprising not merely tumor cells. The heterotypic spheroids that we identified as MUs are
more similar to recently discovered heterotypic spheroids with TAMs at their center (Yin et al., 2016). Both findings emphasize the contribution of ascitic nonepithelial cellular components to spheroid formation and subsequent dissemination of OC. Our results also uncovered the synergistic effect of simultaneous targeting of CAFs and TAMs in hampering OC peritoneal metastasis. Analogously, partial depletion of CAFs significantly decreased the number of metastases in a lung metastatic model (Duda et al., 2010). Fibroblast-associated tumor cell cluster facilitation of tumor growth was also observed in pancreatic ductal adenocarcinoma, ampullary adenocarcinoma, and cholangiocarcinoma (Arnoletti et al., 2018). Intriguingly, stromal fibroblasts already appear in the peritoneal cavity already soon after tumorigenesis, where they recruit exfoliated tumor cells to form MUs, which then adhere and metastasize. Because the PDGFR inhibitor imatinib selectively eradicates fibroblasts and thus disrupts the integrity of MUs, our murine metastasis model. This highlights that MU-driven adhesion occurs early during the metastatic cascade leading to OC dissemination. Unfortunately, clinical trials on the use of imatinib in OC were performed on patients with platinum-resistant, multiple drug–exposed recurrent OC, which likely explains the dismal outcome (Juretzka et al., 2008; Matei

The role of early peritoneal adhesion in OC abdominal metastasis and the notion that exposure to stromal cells can enforce metastatic behavior in neoplastic epithelial cells has increasingly been realized over recent years (Nieman et al., 2011; Pankova et al., 2016). Interestingly, a recent study highlighted that fibroblasts recruited to solid ovarian tumors could be used to detect insidious tumor lesions (Oren et al., 2016). However, stromal fibroblasts already appear in the peritoneal cavity already soon after tumorigenesis, where they recruit exfoliated tumor cells to form MUs, which then adhere and metastasize. Because the PDGFR inhibitor imatinib selectively eradicates fibroblasts and thus disrupts the integrity of MUs, early administration of imatinib dramatically attenuates peritoneal implantation. At a more advanced stage of disease, however, imatinib showed only negligible effects in our murine metastasis model. This highlights that MU-driven adhesion occurs early during the metastatic cascade leading to OC dissemination. Unfortunately, clinical trials on the use of imatinib in OC were performed on patients with platinum-resistant, multiple drug–exposed recurrent OC, which likely explains the dismal outcome (Juretzka et al., 2008; Matei...
Figure 8. **Model of the development of CAF-centered MUs and their role in OC peritoneal dissemination.** During early stages of OC transcoelomic metastasis, tumor cells detach from the primary tumor and meet with activated fibroblasts in the peritoneal cavity. Interactions between tumor cells and CAFs generate specialized compact heterotypic spheroids, which we named MUs of OC for their pivotal role in peritoneal adhesion and invasion. ITGA5 mediates ATCs interaction with CAFs located in the center of MUs, where they provide initial matrix support for OC to avoid anokis. Importantly, activated CAF-secreted EGF is enriched within the MU architecture and specifically induces enhanced ITGA5 expression on ATCs, thus strengthening the integrity of MUs and ultimately promoting exacerbation of OC.

The detailed mechanism underlying spheroid formation between tumor cells or between tumor cells and other ascitic cells was, however, largely unknown. It had been reported that free tumor cells detached from the primary tumor might form spheroids through interactions between α5β1 integrin and fibronectin (Santiago-Medina and Yang, 2016). In the present study, we found that stromal fibroblasts selectively recruited ITGA5\(^{high}\) ATCs. They are thought to initially originate from detached tumor cells that lost their E-cadherin during tumor initiation and progression (Sawada et al., 2008). The inverse correlation between ITGA5 and E-cadherin was reported previously (Sawada et al., 2008) and observed in our study. In addition, tumor cells in the invasive front of primary tumors were demonstrated in our study to present ITGA5\(^{high}\) phenotype, which were more prone to drop into the peritoneal cavity under gravity or intrinsic mechanisms. In this scenario, we therefore ascribed the lack of spheroid formation in LGSOC to the lack of ascitic fibroblasts, rather than the shortage of ITGA5\(^{high}\) ATCs. Blocking ITGA5 drastically attenuates spheroid formation between tumor cells and fibroblasts. Moreover, ITGA5 expression correlates with dismal patient outcome in various cancer types (McKenzie et al., 2013; Ren et al., 2014; Santiago-Medina and Yang, 2016). However, recent clinical trials involving monotherapy with a neutralizing antibody against ITGA5 failed to demonstrate benefit in HGSOC patients (Ricart et al., 2008; Bell-McGuinn et al., 2011). This finding suggests that ITGA5 blockage hampers early MU formation, whereas it might not be effective when the metastatization process is already established. Mechanistically, we demonstrated that tumor cells promote EGF secretion by CAFs within the MU microenvironment that consequently drives ITGA5 expression in ATCs, which in turn further strengthens the tumor–stromal interaction inside MUs. Besides EGF acting as a soluble factor, the increase in EGF-mediated, increased cell–cell contact might also contribute to tumor cell ITGA5 expression through cell-to-cell contact–dependent factors as described previously concerning other integrins (Chandrasekaran et al., 2000; Masszi et al., 2004; Kim et al., 2009b). Early addition of a neutralizing anti-EGF antibody consequently delayed formation of MUs, prevented ITGA5 increase in ATCs, and attenuated peritoneal metastasis in OC xenografts. In parallel, a recent study demonstrated that inhibition of the EGF/EGFR signaling axis reduced spheroid formation between ATCs and macrophages, and eventually attenuated OC tumor growth in a mouse model (Yin et al., 2016). These data not only emphasize the indispensable role of accessory cells such as CAFs in OC metastasis; they also indicate that EGF represents an attractive alternative therapeutic target to interfere with OC dissemination. Possible effects of EGF blockade on normal host cells will have to be explored.

Altogether, this study highlights the unique aggressive nature of the previously underappreciated ITGA5\(^{high}\) ATCs. Stromal CAFs recruit ATCs to form unique compact heterotypic spheroids capable of peritoneal adhesion and metastasis. Our findings thus underscore the pivotal role of MUs in malignant ascites for peritoneal dissemination and define their presence as a major characteristic to discriminate between HGSOC and LGSOC. Furthermore, approaches for the early targeting of stromal CAFs to destroy MUs emerge as new therapeutic strategies to limit HGSOC progression.

### Materials and methods

**Magnetic sorting of tumor cells and fibroblasts from patient-derived tumor tissues**

Human tumor samples were obtained from patients diagnosed with advanced stage of HGSOC and LGSOC who had not received preoperative chemotherapy and for some of whom matched primary tumor, ascites (abdominal washings in LGSOC), and metastases tissues were available (Table S6). All patient tissues were collected from the Department of Gynecological Oncology (Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China) between March 2012 and July 2016 with the informed consent of patients and the authorization of the Ethics Committee of Tongji Hospital and assessed by two senior pathologists. Epithelial tumor cells in tumor tissues of various origins were isolated by the magnetic bead sorting (MACS) system (Miltenyi Biotech). Briefly, for primary and metastatic tumors,
In vivo adhesion assays were performed in the following way: 2 × 10^6 ing cells were counted. The results were presented as proportion of cells were removed by gentle washing with PBS. The remain-
collagen (Merck) and incubated for up to 30 min at 37°C. Nonadher-
substrate and to anti-fibroblast microbeads (Miltenyi Biotech; 130–061-101) for tumor epithelial cell isolation
and viability were checked with calcein (green) and ethidium ho-
response was quantified based on absorption at 490 nm. In vivo adhe-
metastatic tumor cells was normalized to adhesion observed with primary tumor cells.

**Mesothelial clearance assay**
Mesothelial clearance was assessed based on a method described previously (Kenny et al., 2014). Fluorescently labeled HMrSV5 meso-
with ice-cold PBS and sorted with a MACS column (Miltenyi Biotech). The quality of sorting was determined by flow cytometry with a CD326-FITC antibody
trypsin (Thermo Fisher Scientific; ~5 min). Single cells were collected by filtering through cell strainer of 40 μm (BD Biosci-
tach, and imaged at 36 h under a fluorescence microscope (Olym-
pus). To generate heterospheroids and homospheroids, 4 × 10^5 GFP-transfected OC cells were mixed or not with equivalent numbers of PKH-26–labeled primary CAFs and suspended in complete culture medium on ultra-low attachment plates (Corning). Spher-
oids for a subsequent mesothelial clearance test were collected 24–36 h later. To quantify mesothelial clearance, the area covered with GFP-fluorescent spheroids within the blue mesothelial monolayer was measured over time. The data are presented as relative clearance size to that of the control group.

**Patient-derived tumor xenograft model**
After magnetic sorting of epithelial tumor cells from HGSOC tumor samples (primary tumors, ascites fluid, and metastases) and confirmation of the purity of the epithelial component, equivalent numbers of freshly isolated tumor cells (2 × 10^7) from each group were injected i.p. into NOD/SCID mice (n = 6 per group). After ~8 wk, mice in each group were anesthe-
mixed, and micrometastatic nodules were examined throughout the abdominal cavity under the microscope. Furthermore, H&E staining was performed to investigate the formation of invisible xenografts in each group.

**FA formation and cell spreading**
Freshly isolated tumor cells derived from primary tumors, ascites, and metastases were plated on fibronectin (Merck)-coated coverslips and incubated at 37°C for 2 h in complete DMEM/F-12 medium. Formation of FAs was visualized with an anti-paxillin antibody (Abcam; ab32084), and cytoskeleton alterations were detected using rhodamine phalloidin staining (Thermo Fisher Scientific; R415). Immunofluorescence images were acquired, and ImageJ (National Institutes of Health) was used to quantify cell spreading and FA staining. Images were obtained from three independent experiments and combined together. ≥20 cells from each group were analyzed. The total area of each cell was measured with phalloidin staining. FAs were analyzed by measuring the amount of paxillin staining around the cell periphery and normalizing to the cell area.

**Cell culture**
Human OC cell lines SKOV3 and OV90 were purchased from ATCC. Human fibroblast cell line MRC-5 was obtained from the cell bank of the Chinese Academy of Sciences. The human peritoneal mesothelial cell line HMrSV5 was obtained from Jennio Biological Technology. ID8 cells were a gift from the University of Kansas Medical Center. All cell lines were routinely checked for mycoplasma contamination (Lanza) and were authenticated by their source organizations before purchase. All cancer cell lines were maintained in McCoy’s 5A medium containing 10% FBS and 1% penicillin/streptomycin (Thermo Fisher Scientific). MRC-5, HMrSV5, and primary ovarian CAFs were cultured in DMEM/F-12 medium containing 10% FBS and 1% penicillin/streptomycin, at 37°C in a 5% CO2 and 80% humidity incubator. SKOV3 cells had been stably transduced with CMV-Fluc-IRES-RFP lentiviral particles (GeneChem) and designated as SKOV3-Luc, which were further used in animal live imaging experiments.

**In vitro and in vivo adhesion**
In vitro adhesion assays were performed with primary OC cells transiently stained with PKH-67 (Sigma). 2 × 10^4 labeled cells were added into each well coated with 10 μg/ml fibronectin or 10 μg/ml collagen (Merck) and incubated for up to 30 min at 37°C. Nonadher-
test was quantified based on absorption at 490 nm. In vivo adhe-
metastatic tumor cells was normalized to adhesion observed with primary tumor cells.

**Mesothelial clearance assay**
Mesothelial clearance was assessed based on a method described previously (Kenny et al., 2014). Fluorescently labeled HMrSV5 meso-
with ice-cold PBS and sorted with a MACS column (Miltenyi Biotech). The quality of sorting was determined by flow cytometry with a CD326-FITC antibody
trypsin (Thermo Fisher Scientific; ~5 min). Single cells were collected by filtering through cell strainer of 40 μm (BD Biosci-
tach, and imaged at 36 h under a fluorescence microscope (Olym-
pus). To generate heterospheroids and homospheroids, 4 × 10^5 GFP-transfected OC cells were mixed or not with equivalent numbers of PKH-26–labeled primary CAFs and suspended in complete culture medium on ultra-low attachment plates (Corning). Spher-
oids for a subsequent mesothelial clearance test were collected 24–36 h later. To quantify mesothelial clearance, the area covered with GFP-fluorescent spheroids within the blue mesothelial monolayer was measured over time. The data are presented as relative clearance size to that of the control group.

**Patient-derived tumor xenograft model**
After magnetic sorting of epithelial tumor cells from HGSOC tumor samples (primary tumors, ascites fluid, and metastases) and confirmation of the purity of the epithelial component, equivalent numbers of freshly isolated tumor cells (2 × 10^7) from each group were injected i.p. into NOD/SCID mice (n = 6 per group). After ~8 wk, mice in each group were anesthe-
mixed, and micrometastatic nodules were examined throughout the abdominal cavity under the microscope. Furthermore, H&E staining was performed to investigate the formation of invisible xenografts in each group.

**FA formation and cell spreading**
Freshly isolated tumor cells derived from primary tumors, ascites, and metastases were plated on fibronectin (Merck)-coated coverslips and incubated at 37°C for 2 h in complete DMEM/F-12 medium. Formation of FAs was visualized with an anti-paxillin antibody (Abcam; ab32084), and cytoskeleton alterations were detected using rhodamine phalloidin staining (Thermo Fisher Scientific; R415). Immunofluorescence images were acquired, and ImageJ (National Institutes of Health) was used to quantify cell spreading and FA staining. Images were obtained from three independent experiments and combined together. ≥20 cells from each group were analyzed. The total area of each cell was measured with phalloidin staining. FAs were analyzed by measuring the amount of paxillin staining around the cell periphery and normalizing to the cell area.
Transwell invasion assay
Invasion assays were performed according to the manufacturer’s instructions (BD Biosciences). Briefly, Transwell chambers with polycarbonate membrane filters (8-µm pore size; Corning Life Sciences) were coated with 20 µl Matrigel (BD Biosciences) solution diluted in McCoy’s 5A medium (vol/vol 1:4). 2 × 10⁴ OC cells were added to the upper compartment. The lower compartment was filled with McCoy’s 5A medium supplemented with 20% FBS. After 48 h of incubation at 37°C, the upper surface of the filter was washed with PBS and cleared of nonmigratory cells with a cotton swab. The remaining cells at the lower surface of the filter were fixed with cold methanol and stained with 0.1% (wt/vol) crystal violet (Sigma). Invasive cells were scored by counting the whole filter with a microscope at ×200 magnification.

Microarrays and gene expression profiling
After magnetic sorting of tumor epithelial cells from matched primary tumors, ascites and metastases of five HGSOC patients (α1–5) and three LGSOC patients (α01–03), total RNA from these samples was isolated with the RNeasy kit (Qiagen), according to the manufacturer’s protocol. The Human Genome U133 Plus 2.0 Array (Affymetrix) was used for gene expression profiling. Microarray hybridization, washing, staining, and scanning were performed according to standard Affymetrix protocols. The acquired expression data were preprocessed using the MAS5 algorithm to perform background correction and quantile normalization of expression arrays. The random-variance model (RVM) t test and F test were used for analysis of differentially expressed genes between two or more experimental groups, respectively (Wright and Simon, 2003). All hierarchical clustering was performed using average linkage based on Pearson’s correlation of the log₂-transformed expression values, and heat maps were displayed with normalized Z-scores (Miller et al., 2002). The microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus under accession no. GSE73168.

For comparing the profiles of tumor cells that can or cannot form spheroids with CAFs, we isolated heterotypic spheroids immediately (∼4 h) after the development of spheroids by SKOV3 and primary CAFs derived from HGSOC patients (α1–4) in suspension coculture. Next, magnetic sorting was performed in spheroids and individual cells to separate SKOV3 cells, and then subjected to mRNA profiling. Total RNA extracted from spheroid-SKOV3 cells and individual SKOV3 cells was subjected to gene expression profiling with the Human Transcriptome Array 2.0 (HTA2.0) GeneChip. Data normalization and exploring of differentially expressed genes were performed via the Gene-Cloud of Biotechnology Information online tool. The microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus under accession no. GSE98154.

Public database analysis
For analyzing the correlation between ITGA5 expression and the epithelial marker CDH1 in OC, the normalized gene expression data from NCI-60 tumor cells collection (http://discover.nci.nih.gov/cellminer/) and TCGA) datasets were obtained, and Pearson correlation analyses were performed between ITGA5 and CDH1. The prognostic significance of ITGA5 was evaluated by performing a meta-analysis of 2,970 epithelial OC patient expression profiles using the “curatedOvarianData” Bioconductor package. Survival curves were calculated using the Kaplan–Meier method, conducted with the R Bioconductor “survival” package.

Kaplan–Meier analysis
ITGA5 expression was detected in our panel of 160 HGSOC patients based on the available clinical data including time of diagnosis, death, or last follow-up. Overall survival was defined as time from diagnosis to death of OC patients. Patients known to be still alive at time of analysis were censored at the time of their last follow-up. The status of overall survival regarding ITGA5 expression was estimated with the Kaplan–Meier method using GraphPad Prism 6 software, and the log-rank test was used to assess statistical significance.

Suspended spheres formation
Briefly, tumor cells and primary CAFs were collected and washed with cold PBS, then seeded in ultra-low attachment plates (Corning Life Sciences) and cultured in complete medium or malignant ascites supernatant (depleted of cellular components through centrifugation) at 37°C for 48 h. To assess development of heterotypic spheroids, GFP-transfected tumor cells were mixed with PKH-26 (Sigma) labeled primary CAFs (ratio 1:2) in malignant ascites supernatant in ultra-low attachment plates. After overnight culture at 37°C, heterospheroid formation was observed and counted under a fluorescence microscope (Olympus) the next day. The number of cells contained in heterotypic spheroids formed in variant conditions was counted next.

3D invasion assay
Spheroids were embedded in collagen type I (BD) in 96-well plates. The collagen plugs containing the spheroids were incubated in complete DMEM/F-12 medium for various times. Spheroid invasion was observed by fluorescence microscopy. Invasive surface areas were quantified using ImageJ software.

Flow cytometry, cell sorting, cell viability, and anoikis assays
For flow cytometry, all antibodies were purchased already conjugated with fluorescent dyes except the anti-mouse FAP antibody. A PercP-conjugated goat anti-rabbit IgG secondary antibody (Novus; NB7156PCP) was used for mouse FAP detection. To compare variant cell population in ascites of HGSOC and LGSOC patients, ascites samples from HGSOC and LGSOC patients were mildly centrifuged to obtain ascites cells. Then, all ascitic tissues were subjected to RBC lysis buffer (BioLegend) followed by brief trypsinization to dissolve aggregates. Cells were first gated based on forward and side scatter to exclude debris. Dead cells were excluded based on their positive staining for DAPI (Thermo Fisher Scientific; DI306). Ascitic live cells were first gated with APC-conjugated EpCAM (BioLegend; 324208) and PC5.5-conjugated CD45 (BioLegend; 368504). EpCAM CD45⁻ cells were then gated with PE-conjugated CD31 (BioLegend; 303106), and EpCAM CD45⁻ CD31⁻ cells were finally gated with Alexa Fluor 488–conjugated FAP (R&D Systems; FAB3715G) to identify fibroblast percentage.

Gao et al.
Metastatic unit in high-grade serous OC

Journal of Experimental Medicine
https://doi.org/10.1084/jem.20180765
In the orthotopic and i.p. implantation model of GFP-transfected SKOV3 cells, peritoneal heterospheroids were obtained, and GFP+ host cells were sorted on a BD FACS Aria SORP for further identification of variant cell types. The selected GFP+ cells were first gated with PE-conjugated EpCAM (BioLegend; 118206) and APC-conjugated CD45 (BioLegend; 103112). EpCAM ‘CD45−’ cells were then gated with FITC-conjugated CD31 (BioLegend; 102406), and EpCAM ‘CD45−CD31+’ cells were finally gated with PercP-conjugated FAP (Abcam; ab28244) to identify fibroblasts. As for the i.p. implantation model of PKH26-labeled ID8 cells in the EGFP mouse, peritoneal heterospheroids were obtained, and GFP+ host cells were sorted for further identification. The selected GFP+ cells were first gated with PE-conjugated EpCAM and APC-conjugated CD45/CD31 (BioLegend; 103112/102410), and EpCAM ‘CD45−CD31+’ cells were then gated with PercP-conjugated FAP to identify fibroblasts. Corresponding isotype control antibody was used for each antibody in all experiments. All cell samples were run on a FACS Calibur system (Becton Dickinson) and analyzed using CytExpert 2.0 software.

Cell viability was determined using the Cell Counting Kit-8 (Dojindo Laboratories; CCK-8, CK04). For cytotoxicity assays with imatinib (Selleck), 5,000 adherent tumor cells or CAFs per well were plated in 96-well plates and left to adhere before various concentrations of imatinib were applied for 48 h. The CCK-8 solution (10 µl) was added to each well and incubated for another 3 h at 37°C before absorbance at 450 nm was measured on a microplate reader (Bio-Rad). Cell viability was calculated based on the absorbance value relative to untreated control cells. Each assay was performed in triplicate.

For anoikis analysis, 2 × 10^5 single GFP+ SKOV3 cells were allowed to form homospheroids or heterospheroids with CAFs, in the presence or absence of imatinib (20 nM) before being seeded onto 6-well ultra-low attachment plates. After 24 or 48 h, cells were harvested and incubated at 37°C with 0.25% trypsin for 5 min to prevent cell aggregation. Apoptotic GFP+ cells were detected by annexin V-PE (BioLegend; 640908) staining using a FACS Calibur system (Becton Dickinson).

**EGF detection by ELISA**

EGF protein levels in patient malignant ascites and CM of CAFs were measured by ELISA kits (R&D Systems) according to the manufacturer’s protocol. For detection of EGF in spheroids, spheroids were disaggregated with 0.25% trypsin (Thermo Fisher Scientific) in 4% paraformaldehyde (Sigma) for 10 min, permeabilized with 0.25% trypsin for 5 min to prevent cell aggregation. Apoptotic GFP+ cells were detected by annexin V-PE (BioLegend; 640908) staining using a FACS Calibur system (Becton Dickinson).

**Western blot and human antibody array**

Cells were collected and washed with PBS and then lysed with RIPA lysis buffer (Beyotime) supplemented with a protease inhibitor cocktail (Roche). Total protein amount was measured with a bicinchoninic acid assay (Thermo Fisher Scientific), and 40 µg total lysate per sample was subjected to SDS-PAGE followed by immunodetection with the following primary antibodies: ITGA5 (Abcam; ab150361), ITGB1 (Abcam; ab2971), CDH1 (Abcam; ab40772), VIM (Abcam; ab92547), GAPDH (Abcam; ab128915), CK7 (Abcam; ab18598), PDGFRβ (Abcam; ab32570), α-SMA (Abcam; ab7817), and FAP (Abcam; ab28244). For detection, the corresponding HRP-linked secondary antibody (Abcam) and enhanced chemiluminescence (Pierce) were added. Human antibody arrays were performed according to the manufacturer’s instructions. Briefly, human primary CAFs were pretreated with SKOV3-derived CM or 50 ng/ml TGF-β1 (Peprotech) for 48 h, then cultured in serum-free medium for another 48 h. Culture supernatants were collected and assayed using the human cytokine antibody array AAH-CYT-G5 (RayBiotech). Signals were detected using a GenePix 4200A Professional microarray scanner.

**Immunohistochemistry, Masson’s trichrome staining, and immunofluorescence**

OC tissues from primary tumors and metastatic sites in advanced OC patients, as well as normal ovaries and fallopian tubes, were obtained from patients who had undergone surgery at the Department of Gynecological Oncology of Tongji Hospital. Specifically, cancer tissues were from patients diagnosed with advanced (stages III and IV) serous adenocarcinoma based on a pathological evaluation. Normal ovaries and fallopian tubes were obtained from patients who underwent prophylactic adnexectomy due to benign uterine lesions such as multiple adenomyoma. Informed consent was obtained from all patients. Sample sizes were chosen by power analysis. Immunohistochemical staining for ITGA5 in tumor tissues, as well as EGF and EGFR in sequential ovaries, fallopian tubes, primary tumors, and metastases slices were stained as previously described (Greenblatt et al., 2015). Briefly, antigen retrieval was performed in the presence of 0.01 mol/liter EDTA buffer (pH 9.0). After 30 min of blocking in goat serum (Dako), the slides were incubated overnight with primary antibody (ITGA5, ab150361; EGF, ab9695; EGFR, ab52894; Abcam) at 4°C followed by an HRP-linked secondary antibody for 30 min. The slides were then developed using the DAB kit (BD Biosciences) for optimal staining intensity. Three blinded investigators scored the immunostainings based on the staining intensity and the positively stained areas, as previously described (Liu et al., 2014). These data were analyzed as a continuum, and differences between groups were compared with this semiquantitative method.

Masson’s trichrome (Sigma) staining of paraffin-embedded sections of mouse xenografts was performed as previously described (Shen et al., 2014). Dual immunofluorescence for α-SMA (Abcam; ab7817), PDGFRβ (Abcam; ab69506), or prolyl 4-hydroxylase (Thermo Fisher Scientific; MA3-019) with EpCAM (Abcam; ab213500) on HGSOC ascitic spheroids was performed as follows: frozen sections of ascitic tissues were washed with PBS, fixed in 4% paraformaldehyde (Sigma) for 10 min, permeabilized with 0.1% Triton X-100 (Roche), blocked with 5% BSA, stained with a primary antibody for α-SMA/PDGFRβ/prolyl 4-hydroxylase in combination with anti-EpCAM, and then stained with anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 555 (Thermo Fisher Scientific) for 30 min. The nucleus was visualized by staining with DAPI. Representative images were acquired using a fluorescence microscope (Olympus).

**ITGA5 promoter reporter assay**

The human ITGA5 promoter region (−908/+241) was generated by high-fidelity PCR with primer set 5′-CCGCTCGAGGACTG AAGGTGGGTCCT-3′ and 5′-CCGCTCGAGCCGTCTGTTCCGGC GC-3′, using genomic DNA from SKOV3 cells. The PCR product was
digested and cloned into the PGL3 basic vector (Promega) to generate PGL3-ITGA5-Luc construct. Tumor cells grown in 10-cm culture dishes were transiently cotransfected with 10 μg reporter plasmid and 1 μg Renilla luciferase vector (pRL-TK; Promega) for 36 h. Tumor cells were then digested and subjected to spheroid coculture with primary CAFs in variant conditions for 48 h. Subsequently, luciferase activity was evaluated using the dual-luciferase reporter assay system (Promega) in isolated tumor cells, and the data were normalized with Renilla luciferase.

CRISPR/Cas9 editing of ITGA5 and EGFR in cancer cells

Open-access software program CRISPR design was used to design sgRNAs targeting ITGA5 and EGFR. The three highest ranking gRNAs were chosen per gene. Three sgRNA sequences for human ITGA5 are as follows: sgRNA1: 5′-GGGCAACAGTTCAGGCCCA-3′, sgRNA2: 5′-GGAGCAGGCGACACGCCG-3′, and sgRNA3: 5′-TCT GTGCAGGCACTGTGAC-3′. Three sgRNA sequences for human EGFR are as follows: sgRNA1: 5′-GAATTCGCTCACCTGTGTTG-3′, sgRNA2: 5′-TGATCCAAAGCTTCTCCAAC-3′, and sgRNA3: 5′-GAC AGCTTGGATCACTACCTTT-3′. The scrambled sgRNA sequence is 5′-GCCCGAAGCTGGCGAGCAG-3′. sgRNAs oligos were purchased from Vigene Biosciences and then cloned into the Cas9 backbone LentiCRISPR_v2 (Addgene; 52961) according to the manufacturer’s instructions (Ran et al., 2013). Lentivirus production and cancer cell infection were performed as previously described (Taylor et al., 2013). The extent of ITGA5 or EGFR deficiency was first determined by T7E1 assays, as described previously (Kim et al., 2009a). Immunoblotting was then performed in stable pools of ITGA5- or EGFR-deficient SKOV3 cells obtained by culture selection, control or ITGA5/EGFR-deficient SKOV3-Luc cells (5 × 106) were injected either alone or together with control or imatinib-primed CAFs (4 × 106) into the peritoneal cavity of NOD/SCID mice. In the parallel orthotopic metastatic model, SKOV3-Luc cells (4 × 106) were injected orthotopically and randomized to different treatment cohorts, treated i.p. with 100 mg/kg imatinib (in PBS) daily during the initial week (early intervention group), or treated from 1 wk until the end of the experiment (late intervention group). To further analyze TAM depletion influence on OC dissemination, BALB/C nude mice orthotopically inoculated with SKOV3-Luc cells (4 × 106) were randomized to different treatment cohorts, treated i.p. with 100 mg/kg imatinib daily during the initial week (ima group) or treated i.p. with 100 µL LC (FormuMax; 40335E10) every 3 d since tumor inoculation (LC group), or a combination of imatinib and LC as used above (ima+LC group). Control mice received only PBS solution.

All animal experiments were terminated when the mice were restricted in taking in food or drinking water by tumor burdens. Mice were imaged longitudinally with the IVIS Spectrum system (Caliper; Xenogen) 15 min after intraperitoneal administration of 100 mg/kg d-luciferin (Thermo Fisher Scientific). Investigators were blinded for the assessment of the total flux (photons/s) from orthotopic, mesentery, or total peritoneal tumors, which were analyzed using Living Image version 4.3.1 software.

Statistical analysis

All data including error bars are presented as means ± SEM. All calculations were performed using GraphPad Prism 6.0. Two experimental groups were compared by using a paired Student’s t test for paired data or a Student’s t test for unpaired data. Where more than two groups were compared, a one-way ANOVA with Bonferroni’s correction was used. P < 0.05 was considered significant.

Online supplemental material

Fig. S1 depicts ATCs in comparison with matched counterpart tumor cells in LGSOC. Fig. S2 shows the aggressive behavior of CAF-centered spheroids. Fig. S3 depicts fibroblasts contributing to MU formation in various murine models. Fig. S4 shows EGF and EGFR expression pattern in OC tissues. Fig. S5 indicates that early intervention with imatinib or eradication of TAMs prevents peritoneal dissemination in orthotopic mouse models of OC. Tables S1, S2, and S3 show genes significantly dysregulated among ATCs and primary and metastatic tumor cells in HGSOC patients. Table S4 includes genes significantly up-regulated in SKOV3 cells that formed heterotypic spheroids with CAFs compared with those remained individual SKOV3 cells. Table S5 shows cytokine profiles of CM from CAFs in the control group, or from CAFs pre-treated with SKOV3-CM or TGF-β1. Table S6 shows a summary of OC patient clinical data.
References
Ahmed, N., and K.L. Steners. 2013. Getting to know ovarian cancer ascites: opportunities for targeted therapy-based translational research. Front. Oncol. 3:256. https://doi.org/10.3389/fonc.2013.00256
Angela, A.M., D. Cholakian, and A.N. Fader. 2015. Low-grade serous carcinoma: molecular features and contemporary treatment strategies. Expert Rev. Anticancer Ther. 15:893–899. https://doi.org/10.1586/14737440.2015.1052411
Arnoletti, J.P., N. Fanaian, J. Reza, R. Sause, A.J. Almodovar, M. Srivastava, E. Lengyel, R. Palaparthy, K. Gilder, A. Vassos, et al. 2011. A phase II/III, single-arm study of the anti-α5β1 integrin antibody volociximab as monotherapy in patients with platinum-resistant advanced epithelial ovarian or primary peritoneal cancer. J. Clin. Invest. 128:13021–13030. https://doi.org/10.1172/JCI67980
Bapat, S.A., A.M. Mali, C.B. Koppikar, and N.K. Kurrey. 2005. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res. 65:3025–3029. https://doi.org/10.1158/0008-5472.CAN-04-3931
Barbone, D., T.M. Yang, J.R. Morgan, G. Gaudino, and V.C. Broadus. 2008. Mammalian target of rapamycin contributes to the acquired apoptotic resistance of human mesothelioma multicellular spheroids. J. Biol. Chem. 283:13021–13030. https://doi.org/10.1074/jbc.M709698200
Bell-McGuinn, K.M., C.M. Matthews, S.N. Ho, M. Barve, L. Gilbert, R.T. Penson, E. Lengyel, R. Palaparthy, K. Gilder, A. Vassos, et al. 2011. A phase II, single-arm study of the anti-α5β1 integrin antibody volociximab as monotherapy in patients with platinum-resistant advanced epithelial ovarian or primary peritoneal cancer. Gynecol. Oncol. 121:273–279. https://doi.org/10.1016/j.ygyno.2010.12.362
Casey, R.C. and A.P. Skubitz. 2000. CD44 and betai integrins mediate ovarian carcinoma cell migration toward extracellular matrix proteins. Clin. Exp. Metastasis. 18:67–75.
Chandrasekaran, L., C.Z. He, H. Al-Barazi, H.C. Krutzsch, M.L. Iruela-Arispe, and D.D. Roberts. 2000. Cell contact-dependent activation of alpha3beta1 integrin modulates endothelial cell responses to thrombospondin-1. Mol. Biol. Cell. 11:2885–2900. https://doi.org/10.1091/mbc.11.10.2885
Chen, W.J., C.C. Ho, Y.L. Chang, H.Y. Chen, C.A. Lin, T.Y. Ling, S.L. Yu, S.S. Yuan, Y.J. Chen, C.Y. Lin, et al. 2014. Cancer-associated fibroblasts regulate the plasticity of lung cancer stemness via paracrine signalling. Nat. Commun. 5:3472. https://doi.org/10.1038/ncomms4472
Chudecka-Glaz, A.M., A.A. Cymbaluk-Płoska, J.L. Meniskaz, E. Pius-Sadowska, B.B. Machalińska, A. Sompolska-Rzechulia, and I.A. Rzepa-Górsk. 2015. Assessment of selected cytokines, proteins, and growth factors in the peritoneal fluid of patients with ovarian cancer and benign gynecological conditions. OncoFocus Ther. 8:471–485. https://doi.org/10.14214/OTT.53438
Diaz-Padilla, I., A.L. Malpica, L. Minig, L.M. Chiva, D.M. Gershenson, and A. Gonzalez-Martín. 2012. Ovarian low-grade serous carcinoma: a comprehensive update. Gynecol. Oncol. 126:279–285. https://doi.org/10.1016/j.ygyno.2012.04.029
Duda, D.G., A.M. Duyverman, M. Kohno, M. Snuderl, E.J. Steller, D. Fukumura, and G.K. Jain. 2010. Malignant cells facilitate lung metastasis by bringing their own soil. Proc. Natl. Acad. Sci. USA. 107:21677–21682. https://doi.org/10.1073/pnas.100634107
Elmasri, W.M., G. Casagrande, E. Hoskins, D. Kimm, and E.C. Kohn. 2009. Cell adhesion in ovarian cancer. Cancer Treat. Res. 149:297–318. https://doi.org/10.1007/978-3-8091-24
Erez, N., M. Truitt, P. Olson, S.T. Arron, and D. Hanahan. 2010. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. Cancer Cell. 17:135–147. https://doi.org/10.1016/j.ccell.2009.12.041
Greenblatt, M.B., K.H. Park, H. Oh, J.M. Kim, D.Y. Shin, J.M. Lee, J.W. Lee, A. Singh, K.Y. Lee, D. Hu, et al. 2015. CHMP5 controls bone turnover rates by dampening NF-κB activity in osteoclasts. J. Exp. Med. 212:1283–1301. https://doi.org/10.1084/jem.20150407
Huang, C.R., C.T. Lee, K.Y. Chang, W.C. Chang, W.V. Liu, J.C. Lee, and B.K. Chen. 2015. Down-regulation of ARNT promotes cancer metastasis by activating the fibroblast integrin/β1/FAK axis. Oncotarget. 6:11530–11546. https://doi.org/10.18632/oncotarget.4348
Imamura, H., Y. Oishi, M. Aman, K. Shida, T. Shinozaki, N. Yasutake, K. Sonoda, K. Kato, and Y. Oda. 2015. Ovarian high-grade serous carcinoma with a noninvasive growth pattern: simulating a serous borderline tumor. Hum. Pathol. 46:1455–1463. https://doi.org/10.1016/j.humpath.2015.06.002
Juretzka, M., M.L. Hensley, W. Tew, J. Konner, C. Aghajanian, M. Leitao, A. Iasonos, R. Soslow, K. Park, and P. Sabattini. 2008. A phase 2 trial of oral imatinib in patients with epithelial ovarian, fallopian tube, or peritoneal carcinoma in second or greater remission. Eur. J. Gynaecol. Oncol. 29:568–572.
Kenya, H.A., S. Dogan, M. Zillhardt, A. Mitra, S.D. Yamada, T. Krausz, and E. Lengyel. 2009. Organotypic models of metastasis: A three-dimen-
sional culture mimicking the human peritoneum and omentum for the study of the early steps of ovarian cancer metastasis. Cancer Treat. Res. 149:335–351. https://doi.org/10.1007/978-3-8091-24
Kenya, H.A., K.M. Nieman, A.K. Mitra, and E. Lengyel. 2011. The first line of intra-abdominal metastatic attack: breaching the mesothelial cell layer. Cancer Discov. 1:100–102. https://doi.org/10.1158/2159-8290.CD-11-0117
Kenya, H.A., C.Y. Chiang, E.A. White, E.M. Schryver, M. Habis, L.I. Romero, A. Ladanyi, C.V. Penicka, J. George, K. Matlin, et al. 2014. Mesothelial cells promote early ovarian cancer metastasis through fibronectin secretion. J. Clin. Invest. 124:4614–4628. https://doi.org/10.1172/JCI74778
Kenya, H.A., M. Lal-Nag, E.A. White, M. Chen, C.Y. Chiang, A.K. Mitra, Y. Zhang, M. Curtis, E.M. Schryver, S. Bettis, et al. 2015. Quantitative high throughput screening using a primary human three-dimensional organotypic culture predicts in vivo efficacy. Nat. Commun. 6:6220. https://doi.org/10.1038/ncomms7220
Kim, H.J., H.J. Lee, H. Kim, S.W. Cho, and J.S. Kim. 2009a. Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. Genome Res. 19:1279–1288. https://doi.org/10.1101/gr.089471.108
Kim, Y., M.C. Kugler, Y. Wei, K.K. Kim, X. Li, A.N. Brumwell, and H.A. Chapman. 2009b. Integrin alpha3beta1-dependent beta-catatin phosphor-
ylation links epithelial Smad signaling to cell contacts. J. Cell Biol. 184:309–322. https://doi.org/10.1083/jcb.200806067
Kipps, E., D.S. Tan, and S.B. Kaye. 2013. Meeting the challenge of ascites in ovarian cancer: new avenues for research and therapy. Nat. Rev. Cancer 13:273–282. https://doi.org/10.1038/nrc3432

Koncny, G.E., C. Wang, H. Hamidi, B. Winterhoff, K.R. Kalll, J. Dering, C. Giner, H.W. Chen, S. Dowdy, W. Cliby, et al. 2014. Prognostic and therapeutic relevance of molecular subtypes in high-grade serous ovarian cancer. J. Natl. Cancer Inst. 106:djz249. https://doi.org/10.1093/jnci/djz249

Lane, D., I. Matte, C. Rancourt, and A. Piche. 2011. Prognostic significance of IL-6 and IL-8 ascs levels in ovarian cancer patients. BMC Cancer 11:270. https://doi.org/10.1186/1471-2407-11-270

Latifi, A., R.B. Luwor, M. Bilandzic, S. Naazarian, K. Steners, J. Pyman, H. Zhu, E.W. Thompson, M.A. Quinn, J.K. Findlay, and N. Ahmed. 2012. Isolation and characterization of tumor cells from the ascites of ovarian cancer patients: molecular phenotype of chemoresistant ovarian tumors. PLoS One. 7:e46858. https://doi.org/10.1371/journal.pone.0046858

Lawrenson, K., D. Sproul, B. Grun, M. Notaridou, E. Benjamin, I.J. Jacobs, D.filtration and the participation of MMP2. Int. J. Cancer. 114:531–543. https://doi.org/10.1002/ijc.20778

Liu, D., L. Li, X.X. Zhang, D.Y. Wan, B.X. Xi, Z. Hu, W.C. Ding, D. Zhu, X.L. Ren, J., S. Xu, D. Guo, J. Zhang, and S. Liu. 2014. Increased expression of αβ1 integrin is a prognostic marker for patients with gastric cancer. Clin. Trans. Oncol. 16:668–674. https://doi.org/10.1007/s11585-013-1133-y

Ricart, A.D., A.W. Tolcher, G. Liu, K. Holen, G. Schwartz, M. Albertini, G. Weiss, S. Yazi, C. Ng, and G. Wilding. 2008. Veloxximab, a chimeric monoclonal antibody that specifically binds alphabeta1 integrin: a phase I, pharmacokinetic, and biological corollary study. Clin. Cancer Res. 14:7924–7929. https://doi.org/10.1158/1078-0432.CCR-08-0378

Rizvi, I., U.A. Gurkan, S. Tasoglu, N. Alagic, J.P. Celli, L.B. Mensah, Z. Mai, U. Demirci, and T. Hassan. 2013. Flow induces epithelial-mesenchymal transition, cellular heterogeneity and biomarker modulation in 3D ovarian cancer nodules. Proc. Natl. Acad. Sci. USA. 110:E1974–E1983. https://doi.org/10.1073/pnas.1216699110

Santiago-Medina, M., and J. Yang. 2016. MENA promotes tumor-intrinsic metastasis through ECM remodeling and haptotaxis. Cancer Discov. 6:474–476. https://doi.org/10.1158/2159-8290.CD-16-0231

Sawada, K., A.K. Mitra, A.R. Radjabi, V. Bhaskar, E.O. Kistner, M. Tretiakov, J. Jagadeeswaran, A. Montag, A. Becker, H.A. Kenny, et al. 2008. Loss of E-cadherin promotes ovarian cancer metastasis via alpha 5-integrin, which is a therapeutic target. Cancer Res. 68:2339–2339. https://doi.org/10.1158/0008-5472.CAN-07-5167

Schmeler, K.M., and D.M. Gershenson. 2008. Low-grade serous ovarian cancer: a unique disease. Curr. Oncol. Rep.10:519–523. https://doi.org/10.1007/s11991-008-0089-9

Shen, K., S. Luk, D.F. Hicks, J.S. Elman, S. Bohr, Y. Iwamoto, R. Murray, K. Pena, F. Wang, E. Seker, et al. 2014. Resolving cancer-stroma interfacial signalling and interventions with micropatterned tumour-stromal assays. Nat. Commun. 5:4662. https://doi.org/10.1038/ncomms5662

Sodek, K.L., M.J. Ringueste, and T.J. Brown. 2009. Compact sphere formation by ovarian cancer cells is associated with contractile behavior and an invasive phenotype. Int. J. Cancer. 124:2060–2070. https://doi.org/10.1002/ijc.24188

Suzuki, N., A. Higashiguchi, Y. Hasegawa, H. Matsumoto, S. Oie, K. Orikawa, S. Ezawa, N. Susumu, K. Miyashita, and D. Aoki. 2005. Loss of integrin α3β1 expression associated with acquisition of invasive potential by ovarian clear cell adenocarcinoma cells. Hum. Cell. 18:147–155.

Taylor, M.A., K. Sossey-Alaoui, C.L. Thompson, D. Danielpour, and W.P. Schie mann. 2013. TGF-β upregulates miR-18a expression to promote breast cancer metastasis. J. Clin. Invest. 123:150–163. https://doi.org/10.1172/JCI84946

Thibault, B., M. Castells, J.P. Delord, and B. Coudier. 2014. Ovarian cancer microenvironment: implications for cancer dissemination and chemoresistance acquisition. Curr. Metastasis Rev. 33:37–39. https://doi.org/10.1007/s10555-013-9456-2

Tung, C.S., S.C. Mok, Y.T. Tsang, Z. Z. Hu, S. Song, J. Liu, M.T. Deavers, A. Malpica, J.K. Wolf, K.H. Lu, et al. 2009. PAX2 expression in low malignant potential ovarian tumors and low-grade serous ovarian carcinomas. Mod. Pathol. 22:1243–1250. https://doi.org/10.1038/modpathol.2009.92

Vaughan, S., J.I. Coward, R.C. Bast Jr., A. Berchuck, J.S. Berek, J.D. Brenton, G. Tihan, J. Sen, C. Caffery, O. E. Gauduchon, and F. Carreiras. 2005. Transmigration of human ovarian carcinoma cells through endothelial extracellular matrix involves alpha 5-integrin and the participation of MMP2. Int. J. Cancer. 114:531–543. https://doi.org/10.1002/ijc.20778

Zawalich, E. W . Thompson, M.A. Quinn, J.K. Findlay, and N. Ahmed. 2012. Iso-