Integrin αvβ3 and Fibronectin Upreregulate Slug in Cancer Cells to Promote Clot Invasion and Metastasis

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Abstract

The blood clotting cascade is selectively involved in lung metastasis, but the reason for this selectivity is unclear. Here, we show that tumor cells that metastasize predominantly to the lung, such as renal cell carcinoma (RCC) and soft tissue sarcoma (STS), have an inherent capacity to generate extensive invadopodia when embedded in a blood clot. Compared with other metastatic cancer cells tested, RCC and STS cells exhibited increased levels of expression of fibronectin and an activated form of the integrin αvβ3, which coordinately supported the generation of an elaborate fibronectin matrix and actin stress fibers in fibrin-embedded tumor cells. Together, fibronectin and αvβ3 induced upregulation of the transcription factor Slug, which mediates epithelial–mesenchymal transition as well as fibrin invasion and lung metastasis. This mechanism is clinically significant, because primary cancer cells from patients with metastatic RCC strongly invaded fibrin and this correlated with fibronectin matrix formation and Slug expression. In contrast, tumor cells from patients with localized RCC were largely noninvasive. Together, our findings establish that activated integrin αvβ3 and fibronectin promote lung metastasis by upregulating Slug, defining a mechanism through which cancer cells can colonize blood clots in the lung vasculature. Cancer Res; 73(20); 6175–84. ©2013 AACR.

Introduction

Metastasis to distant organs is a common complication of malignant tumors (1). Although organ-confined primary tumors can be cured by surgical excision, the overwhelming majority of patients with metastases has an extremely poor prognosis. Resection of solitary metastases, immune therapy as well as treatment with targeted drugs designed to block growth and survival pathways has had some success; however, after a phase of remission, patients typically suffer disease recurrence and progression (2, 3). These outcomes warrant further mechanistic studies toward the identification of novel targets for more efficient antimetastatic treatments.

The metastatic cascade begins at the site of the primary tumor from where tumor cells are shed into the blood circulation (1). After entering the blood stream, tumor cells attach in the vasculature of distant organs and extravasate into the perivascular tissue, where they either begin to proliferate and form metastases or become dormant. Although the initial tumor cell attachment does not require clotting activity, thrombus formation becomes relevant in the subsequent steps of metastatic seeding by promoting invasive functions that enable tumor cell extravasation and by protecting tumor cells from circulating immune cells (4, 5). Moreover, it has been shown that blood clot provides a microenvironment that is crucial for maintenance and proliferation of tumor-initiating cells (6). Conversely, inhibiting clotting with anticoagulants or knocking down critical coagulation and platelet factors in transgenic mice (e.g., tissue factor, fibrinogen, Gtoq) has a marked antimetastatic effect, indicating that the generation of a fibrin platelet thrombus plays a key role for metastasis (7, 8).

In addition to host factors, the capacity of tumor cells to metastasize requires a specific set of tumor cell functions including the ability to invade as a single cell (9). These specific promigratory and proinvasive functions are induced by transcriptional programs that mediate epithelial–mesenchymal transition (EMT) in tumor cells (9). EMT is regulated by a set of transcription factors, most notably E-cadherin, and upregulation of mesenchymal markers such as vimentin, N-cadherin, fibronectin, and integrins (10). EMT can be initiated by oncogenes, hypoxia, as well as a number of soluble factors including TGF-β and Wnt (11–14). In addition, it has been shown that integrins typically upregulated in cancer such as αvβ3 can promote EMT through degradation of E-cadherin and activation of intracellular signaling cascades (15).

Integrin αvβ3 has been detected in tumor tissues from patients with melanoma, breast cancer, and pancreatic cancer, and its expression is particularly pronounced in metastatic tissue (16–18). Tumor cells that express integrin αvβ3 metastasize aggressively, and blocking integrin αvβ3 with antibodies,
Arg-Gly-Asp (RGD)-containing peptides, or siRNA inhibits this process (5, 16, 19). Metastasis mediated through \( \alpha v \beta 3 \) has been shown to depend on integrin activation, suggesting that \( \alpha v \beta 3 \) function is controlled on the transcriptional as well as post-translational level (20). Using plasma fibronectin-deficient mice, we previously showed that \( \alpha v \beta 3 \)-mediated lung metastasis depends on fibrin–fibronectin complexes, which facilitate invadopodia formation in clot-embedded tumor cells by activating integrin \( \alpha v \beta 3 \) (5). To determine if there is a correlation between lung metastasis and clot invasion, we assessed the capacity of human tumor cells to generate invadopodia in clotted plasma and tested if invadopodia formation in clotted plasma is governed by mechanisms relevant for lung metastasis.

Materials and Methods

Tumor cells

RCC4, 786-0, CAKI-1 (renal cell carcinoma, RCC), HT1080, RD (soft tissue sarcoma, STS), U87MG (glioblastoma), MDA-MB-231, MCF-7 (breast cancer), A375 (melanoma), PANC1 (pancreatic cancer), PC-3, and LNCaP (prostate cancer) cell lines were purchased from the American Type Culture Collection and cultured according to the manufacturer’s specifications for less than 6 months. Primary human tumor cells were isolated from patients with metastatic and localized RCC (Supplementary Table S1). Immediately following resection, tumors were inspected by an anatomical pathologist and harvested under sterile conditions, then de-identified by the tissue bank of the University of Pittsburgh Cancer Institute. Upon receipt in the laboratory, the tissue was cut into small pieces, washed once with sterile PBS, and digested with 2.4 U/ml dispase II (Roche) at 37 °C for 50 minutes on a rotator. The suspension was centrifuged at 1,100 rpm for five minutes at 4 °C and the pellet was resuspended in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS. Cells were cultured at 37°C under a humidified, 5% CO2 atmosphere before cryopreservation.

siRNA-mediated gene silencing

The 786-0 and HT1080 cells were grown for 24 hours before transfection with 25 nmol/L integrin \( \beta 3 \) (Dharmacon On-TARGETplus SMARTpool L-040746-01), integrin \( \beta 1 \) (Dharmacon On-TARGETplus SMARTpool L-004506-00), FN (Dharmacon On-TARGETplus SMARTpool L-009853-00), Slug (Dharmacon On-TARGETplus SMARTpool L-017386-00), or nontargeting control (Dharmacon On-TARGETplus D-001810-10) siRNA. SMARTpool siRNA contains a pool of four siRNA sequences directed against the target gene. Cells were transfected in Opti-MEM medium (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer instructions. After five hours, cells were placed in normal culture medium and grown for an additional 43 hours. Target knockdown was confirmed by Western blot analysis.

In vivo assays

To induce metastasis, \( 5 \times 10^5 \) HT1080 cells were injected into the tail vein (i.v.) of female, 6- to 8-week-old athymic nude mice (Charles Rivers). Four weeks after tumor cell injection, lungs were isolated, fixed in Bouin’s solution (Sigma), and weighed. Tumor burden was assessed by subtracting the weight of tumor-free lungs (0.22 g ± 0.006) from the weight of tumor-bearing lungs. To assess tumor multiplicity, tumor nodules were counted on the surface of lungs using a stereo microscope (Zeiss Stemi 2000-C). To inhibit thrombin function, we injected 500 IU hirudin (Millipore) together with the tumor cell suspension. To inhibit integrin \( \beta 3 \) and Slug, HT1080 cells were transfected with siRNA 48 hours before intravenous injection. To analyze colocalization, mice were intravenously injected with cytotracker green-labeled HT1080, 786-0, MDA-MB-231, or A375 cells (Invitrogen) and perfused through the heart after one hour. Lung tissue was snap frozen in optimum cutting temperature compound after isolation. Frozen tissues were sectioned, fixed with acetone, and stained with biotinylated mouse fibrinogen antisera (Nordic), followed by streptavidin Alexa Fluor 594 (Invitrogen). Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI)-containing mounting media ( Vectashield). Tissues were analyzed at \( \times 20 \) magnification using a fluorescence microscope (Zeiss Axiosplan 2), and scored for colocalization of tumor cells with fibrin.

Three-dimensional cell culture

Clot embedding was carried out as previously described (5). Briefly, tumor cells were mixed either with citrated human plasma (US Biological) in the presence of 4.5 mmol/L CaCl2 to generate clotted plasma or with 2 mg/mL fibrinogen (Enzyme Research Laboratories, Inc.) in the presence of 2 mmol/L CaCl2 and 25 µg/mL FXIII (Enzyme Research Laboratories) to generate fibrin gels. Clotting was induced with 2.5 U/mL thrombin (Sigma), and 15 µL suspensions were pipetted onto tissue culture plates and inverted at room temperature for 10 minutes to solidify. For Matrigel studies, cells were mixed with ice-cold Matrigel (BD Biosciences) and then incubated at 37°C for 10 minutes to solidify the gel. Embedded cells were incubated with media supplemented with 10% FBS at 37°C under a humidified, 5% CO2 atmosphere.

Invadopodia analysis

Clots or matrigel plugs were analyzed for invadopodia formation 24 and 48 hours after embedding at designated areas by phase-contrast microscopy (Nikon Eclipse TS100; \( \times 10 \) magnification). Invadopodia formation was classified as complete (i.e., elongated or stellate shape) or incomplete (i.e., round shape with or without rudimentary invadopodia) and calculated for completely spread cells as percentage of the total cell number of a microscopy field. Colonization was measured by counting the total number of clot-embedded cells per microscopic field. In addition, we analyzed invadopodia formation over time by live cell imaging. To this end, fibrin embedded 786-0 or MDA-MB-231 cells were transferred to a BioStation IM (Nikon) and pre-equilibrated to 37°C and 5% CO2. Phase-contrast images were captured every 5 minutes for one hour and then every 30 minutes for the next 71 hours to track invadopodia. Images and videos were prepared using the BioStation IM software. Using these images, the total number of invadopodia were counted and divided by the total number of cells in each of four fields to yield the average number of...
invadopodia per cell for each time point across the first 24 hours. Using ImageJ software, the length of each invadopodium in each of four fields was measured and an average calculated for each time point across the first 24 hours. For each cell without invadopodia, a zero was calculated into the average.

**Confocal microscopy**

Fibrin-embedded tumor cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% triton × 100, and incubated with anti-fibronectin (Millipore) or isotype control followed by incubation with Alexa Fluor 488-conjugated secondary antibody (Invitrogen), and analyzed using a confocal microscope (Leica TCSSL). To visualize the cytoskeleton and nuclei, cells were stained with Alexa Fluor 546-phalloidin (Invitrogen) and Draq5 (eBioscience), respectively. Digitized images were processed with Adobe Photoshop.

**Western blot analysis**

Cell pellets were lysed by adding 2× SDS sample buffer. Proteins were separated by SDS–PAGE, transferred onto polyvinylidene difluoride (PVDF), and stained with 0.05% Ponceau S (Sigma) to ensure equivalent protein loading. Immunoblots were blocked with 5% BSA and probed overnight at 4°C with antibodies against integrin β3 (BD Biosciences), fibronectin (EMD Millipore), vimentin, E-cadherin, N-cadherin, Twist, Snail, and Slug (Cell Signaling Technology). Immunoreactivity was detected using peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody and visualized by enhanced chemiluminescence.

**Cell adhesion assay**

Cell adhesion was measured as previously described (21). Forty-eight-well plates (Costar, polystyrene, non-tissue culture treated) were coated with fibronogen at 4°C overnight, then washed with PBS, and blocked with 0.1% BSA (one hour, 37°C). Cells were suspended in HEPES-Tyrode’s buffer containing 0.1% BSA and 1 mmol/L MgCl₂, added to the plate at 1 × 10⁵ in 200 µL/well and allowed to attach for up to 60 minutes (37°C, 5% CO₂). Plates were washed to remove floating cells and the remaining attached cells were incubated with para-nitrophenol phosphate (5 mg/mL in 50 mmol/L sodium acetate, 1% Triton X-100, pH 5.2) for 30 minutes and quantified at 405 nm after adding 0.3 mol/L sodium hydroxide.

**Flow cytometry assay**

Tumor cells were suspended in HEPES-Tyrod’s buffer containing 0.1% BSA, 0.4 mmol/L CaCl₂, 1 mmol/L MgCl₂, and then incubated with antibody specific for activated αvβ3 (wow1) for 30 minutes (22). Manganese sulfate (0.25 mmol/L) or EDTA (10 mmol/L) was added as positive and negative controls. Cells were then washed with ice-cold buffer, and incubated for 30 minutes on ice with Alexa Fluor 488 anti-mouse F(ab’)2 (Invitrogen). Cell viability was monitored by staining cells with 5 µg/mL propidium iodide (Roche Applied Science). Fluorescence was examined in FL1 on 10,000 viable cells per sample using a tabletop cytometer (Accuri C6). Data are shown as percentage of positive cells by setting a marker (M1) at 5% of control-labeled cells (secondary antibody only).

**Statistical analysis**

Data were analyzed using unpaired two-tailed Student t test or one-way ANOVA followed by the post hoc Tukey multiple comparisons test (GraphPad Prism 5). Treatment differences with a two-sided P value less than 0.05 were considered significantly different. Error bars show mean ± SEM.

**Results**

**Tumor cell seeding into the lung is associated with clot formation**

Tumor cells have been shown to express procoagulant factors that can activate the clotting cascade (23). To assess the role of clot formation for lung metastasis, we analyzed tissue sections from lungs isolated 1 hour after tail-vein injection with a panel of fluorescence-labeled tumor cell lines derived from RCC, STS, as well as melanoma and breast cancer by fluorescence microscopy. Immunohistochemistry for fibrin, which is a major clot component, revealed that a large majority of tumor cells were surrounded by an extensive meshwork of clot that formed early during lung seeding and was independent of the tumor type (Fig. 1A and B). Scoring lung tissue sections for the ratio of tumor cells colocalizing with fibrin, we found a positive association in more than 80% of 786-0 RCC, HT1080 STS and MDA-MB-231 breast tumor cells and in more than 40% of A375 melanoma cells. To determine the role of clotting for lung metastasis, we injected mice with a single dose of the thrombin inhibitor hirudin at the time of HT1080 tumor cell injection. Inspection of lungs four weeks later showed that tail-vein injection of HT1080 resulted in extensive tumor burden of lungs in the control cohort, whereas metastasis was markedly reduced in the cohort that received the clotting inhibitor (Fig. 1C and D). Together, our results indicate that tumor cells in the lung are surrounded by blood clot and that the generation of blood clot is relevant for tumor cell seeding in the lung.

**Invadopodia analysis of clot-embedded tumor cells**

We previously showed that the capacity of murine tumor cells to generate invadopodia in clotted plasma correlates with increased lung metastasis (5). To study the role of invadopodia formation in human tumor cell models, we embedded a panel of cell lines derived from RCC (786-0, RCC-4, CAKI1), STS (HT1080, RD), glioblastoma (U87MG), breast cancer (MDA-MB-231, MCF-7), prostate cancer (PC-3, LNCaP), melanoma (A375), and pancreatic cancer (PANC1) in a three-dimensional (3D) matrix of clotted plasma. We inspected the plasma clots after 24 and 48 hours by phase-contrast microscopy and found that a significant fraction of the plasma clot-embedded RCC, STS, and glioblastoma cells featured a spread phenotype with extensive invadopodia (20–60% of cells; Fig. 2A and B). This is in stark contrast to the phenotype of a random panel of breast, prostate, melanoma, and pancreatic tumor cell lines that displayed a ratio of spread, invadopodia-positive cells to round, invadopodia-negative cells of less than 10% (Fig. 2A and B). To further analyze the adhesive mechanisms guiding invadopodia formation, we embedded the clot-invasive tumor cell lines in fibrin or matrigel. Interestingly, while these cells maintained their capacity to generate invadopodia in fibrin, which is the
main component of clotted plasma, most of them showed only limited ability to spread in matrigel (Fig. 2C), suggesting that clot invasion is mediated by a specific set of adhesive cell functions that facilitate interaction with fibrin. Together, our results indicate that our panel of RCC, STS, and glioblastoma cells represents a specific fibrin-interactive phenotype that promotes invadopodia formation in a 3D matrix of clotted plasma.

Invadopodia formation in clot-embedded tumor cells correlates with upregulation of integrin αvβ3 and fibronectin

To analyze clot invasion in more detail, we carried out live cell imaging of 786-O RCC cells, which began to generate invadopodia within the first hour of embedding in fibrin (Fig. 3A and B and Supplementary Movie S1 and S2). The spread appearance of 786-0 cells was maintained even when cells began to form colonies (24–48 hours), which later clustered into large strands (48–72 hours). Fibrin-embedded MDA-MB-231 cells, on the other hand, developed smaller sized invadopodia more sporadically and at a later time point (Fig. 3A–C and Supplementary Fig. S1A). Together, these results describe significant differences in invadopodia dynamics between distinct tumor cell types.

On the basis of the mesenchymal phenotype of fibrin-embedded 786-0 cells, we decided to test expression of markers and transcription factors involved in EMT. Besides overall upregulation of mesenchymal markers (vimentin, N-cadherin, Twist, Snail, and Slug) and loss of the epithelial marker E-cadherin, we found strong expression of β3 integrin and fibronectin in RCC, STS, and glioblastoma cells, which correlated closely with their ability to generate invadopodia in clotted plasma and fibrin (Fig. 3D). To further define β3 expression on tumor cells, we conducted flow cytometry with an antibody, LM609, that specifically recognizes the αvβ3 integrin dimer. These data confirm that the RCC, STS, and glioblastoma cells express overall higher levels of integrin αvβ3 than the other tumor cell lines that were unable to generate invadopodia in clotted plasma (Supplementary Fig. S1B). Together, our results indicate that overexpression of integrin αvβ3 and fibronectin correlates with a mesenchymal phenotype of clot-embedded tumor cells and their ability to generate extensive invadopodia.

Invadopodia-generating tumor cells express activated integrin αvβ3

We previously showed that integrin αvβ3 promotes invadopodia formation and lung metastasis in a murine melanoma model (5). Paralleling these results, we found that knocking down integrin αvβ3 with siRNA or short hairpin RNA (shRNA) significantly inhibited invadopodia formation in human 786-0 and HT1080 cells embedded in fibrin whereas siRNA against β1 integrins had no such effects (Fig. 4A and Supplementary Fig. S2A and S2B). In addition, we saw a significant negative effect of β3 siRNA/shRNA on tumor cell proliferation and colony formation (Fig. 4A and Supplementary Fig. S2C and S2D). Transient knockdown of αvβ3 in HT1080 cells with siRNA was also sufficient to reduce experimental lung metastasis, suggesting that integrin αvβ3-mediated functions are necessary in the initial phase of lung colonization, when clotting plays an important role for the tumor cell fate (Fig. 4B).

Figure 1. Tumor cell seeding in the lung is associated with clot formation. A and B, lungs from athymic nude mice were isolated immediately (control; no injection) or one hour after i.v. injection with Cytotracker-labeled HT1080, 786-0, MDA-MB-231, and A375 cells (green) and analyzed for colocalization with fibrin (red). A, representative images (scale bar, 50 μm). Nuclei were stained with DAPI (blue). B, fluorescence microscopy images were scored for tumor cells that colocalize with fibrin as percent of total tumor cell count per optical field [Fib' TC (%)]. C and D, panel of lungs harvested four weeks after i.v. injection of 5 × 10^6 HT1080 fibrosarcoma cells in the presence of 500 IU hirudin (D, bottom) or vehicle (D, top) was analyzed for tumor burden (C; lung weight in grams). ***, P < 0.001 versus vehicle.
further assess the role of integrin \( \alpha v \beta 3 \) for invadopodia formation, we labeled our tumor cell panel with wow1 antibody, which binds only activated integrin \( \alpha v \beta 3 \) (22). Using flow cytometry, we detected wow1-binding as an indicator of \( \alpha v \beta 3 \) activation on each of the invadopodia-positive tumor cells but only on one out of 4 invadopodia-negative tumor cell lines (Fig. 4C and D). In addition, the functional difference of \( \alpha v \beta 3 \) was detectable when we tested cell attachment to the fibrin-precursor fibrinogen, a plasma adhesion protein that specifically interacts with activated \( \alpha v \beta 3 \) but not with the resting integrin (21). This experiment showed that invadopodia-positive tumor cells spontaneously adhere and spread on fibrinogen, whereas most of the invadopodia-negative tumor cells are unable to attach unless manganese was added for \( \alpha v \beta 3 \) activation (Fig. 4E and F; Supplementary Fig. S3). An exception from this pattern is represented by the invadopodia-negative A375 cells, which bound wow1 and adhered spontaneously but were unable to spread on fibrinogen (Fig. 4D and E and Supplementary Fig. S3). Together, these results indicate that expression of activated integrin \( \alpha v \beta 3 \) on tumor cells is a prerequisite for invadopodia formation and lung metastasis.

Integrin \( \alpha v \beta 3 \) promotes fibronectin matrix formation in invadopodia-forming tumor cells

Fibronectin matrix formation has been shown to depend on integrin activation (24). In line with this, we found that fibrin embedded 786-0 and HT1080 cells, which both express activated \( \alpha v \beta 3 \), generated an elaborate fibronectin matrix whereas MDA-MB-231 cells, which only expressed inactive \( \alpha v \beta 3 \), were unable to do so (Fig. 5A and C). Moreover, fibronectin matrix formation was strongly reduced in 786-0 cells transfected with siRNA against \( \beta 3 \) integrin compared with transfection with integrin \( \beta 1 \) siRNA, indicating that \( \alpha v \beta 3 \) plays a pivotal role in organizing the extracellular matrix in clot-embedded cells (Fig. 5A and C). To further delineate the interaction between fibronectin and integrin \( \alpha v \beta 3 \) in fibrin-embedded tumor cells, we analyzed F-actin dynamics in 786-0 cells fluorescently labeled with phalloidin (Fig. 5B and D). Using confocal microscopy, we identified extensive stress fibers that were associated

Figure 2. Invadopodia analysis of clot-embedded tumor cells. A and B, tumor cells were embedded in a three-dimensional matrix of clotted plasma and analyzed for invadopodia formation by phase-contrast microscopy. A, representative images of clot-embedded tumor cells after 48 hours (top, left to right: 786-0, RCC4, CAKI-1, RD, HT1080, U87MG; bottom, left to right: MDA-MB-231, MCF-7, PC3, LNCaP, A375, PANC-1). Scale bar, 50 \( \mu m \). B, phase-contrast microscopy images of plasma clots were scored for tumor cells with invadopodia as percentage of total tumor cell count per optical field 24 and 48 hours after embedding. C, invadopodia formation in fibrin and Matrigel 48 hours after embedding (invadopodia-positive cells as percentage of total).

Figure 3. Invadopodia formation in clot-embedded tumor cells correlates with upregulation of integrin \( \alpha v \beta 3 \) and fibronectin. A–C, phase-contrast images were captured at indicated times to track invadopodia formation using the BioStation IM live microscopy. A, representative images of 786-0 and MDA-MB-231 cells 1, 24, 48, and 72 hours after embedding in fibrin. Scale bar, 20 \( \mu m \). B and C, the number (B) and length (C) of invadopodia per cell (786-0 vs. MDA-MB-231) is shown at indicated time points. **, \( P < 0.05 \); ***, \( P < 0.01 \); ****, \( P < 0.001 \) of 786-0 versus MDA-MB-231. D, total cell extracts from invadopodia-positive or invadopodia-negative tumor cell lines were immunoblotted for \( \beta 3 \) integrin (\( \beta 3 \)), fibronectin (FN), vimentin, E-cadherin, N-Cadherin, Twist, Snail, and Slug. Ponceau S (PS) staining shows protein loading.
Integrin αvβ3 and fibronectin cooperate to maintain Slug expression

The interaction of integrins with the extracellular matrix is an important mediator of EMT (15). To determine the role of integrin αvβ3 and fibronectin for tumor cell EMT, we collected extracts from siRNA-treated 786-0 and HT1080 cells. Interestingly, Western blot analysis of the extracts showed a specific reduction of the EMT transcription factor Slug after β3 as well as fibronectin knockdown whereas other EMT transcription factors such as Snail and Twist remained unchanged (Fig. 6A). Transfection with Slug siRNA, in turn, inhibited invadopodia formation in clot in vitro and experimental lung metastasis in vivo although fibronectin and β3 integrin expression remained unchanged (Fig. 6B–D; Supplementary Fig. S5A and B). In addition to fibronectin and integrin αvβ3, we found the TGF-β/smooth muscle α actin pathway consistently activated in clot-invasive tumor cells. However, treatment with the TGFBR1 inhibitor SB431542 had no effect on Slug expression, suggesting that αvβ3 and fibronectin act independently of the TGF-β pathway (Supplementary Fig. S5C and D).

To follow up on these findings, we analyzed primary kidney tumor cells from patients with metastatic and nonmetastatic disease for β3, fibronectin, and Slug expression (Fig. 7A and Supplementary Table S1). Although we found αvβ3 expressed in metastatic as well as nonmetastatic cells, we saw a striking difference with respect to invadopodia formation, fibronectin expression, and fibronectin matrix formation, which all were extensive among the metastatic RCC cells but barely detectable in nonmetastatic RCCs (Fig. 7A–D). While cells from metastatic tumor #3 and nonmetastatic tumor #4 invaded to a lesser extent compared with the metastatic cell lines #1 and #2, these cells also expressed lower levels of Slug than the other metastatic cell lines. Paralleling these results, we found that cell lines #1 and #2 are derived from patients with lung metastasis whereas patient #3 suffered from metastases to the bone and adrenal gland (Supplementary Table S1). Taken together, these results indicate a direct link between Slug expression, invadopodia formation, and lung metastasis.

Discussion

We previously showed that clot formation promotes lung metastasis but not metastasis to the liver (5). Here, we show that tumor cells from cancers that predominantly metastasize to lungs, such as RCC and STS (25, 26), generate extensive invadopodia in clotted plasma and fibrin. Invadopodia formation in these cells is mediated by activated integrin αvβ3, fibronectin, and Slug, which in turn promotes experimental lung metastasis in vivo. The correlation between Slug expression, fibronectin matrix formation, and fibrin invasion was confirmed in primary tumor cells recently isolated from this function, indicating that fibronectin supports invadopodia formation downstream of the pathways that govern integrin αvβ3 activation (Fig. 5F). Together, our results show that integrin αvβ3 supports fibronectin matrix formation and that this interaction is critical for spreading and proliferation of fibrin-embedded tumor cells.

with a well-organized meshwork of fibronectin fibrils in control-transfected cells. The ability to generate stress fibers remained intact in cells transfected with integrin β1 siRNA but was markedly impaired when we knocked down fibronectin or integrin αvβ3. These results suggest a prominent role of integrin αvβ3 and fibronectin for spreading in fibrin. In support of this concept, our results show that inhibiting fibronectin expression with siRNA or shRNA impairs invadopodia formation and proliferation of fibrin-embedded 786-0 and HT1080 cells in a manner similar to inhibiting integrin αvβ3 (Fig. 5E; Supplementary Fig. S4). In contrast to knocking down αvβ3, which strongly diminished tumor cell adhesion to fibrinogen, treatment with fibronectin siRNA had no effect on
patients with metastatic RCC, suggesting that our finding is clinically relevant.

Extravasation of tumor cells from the blood circulation to the interstitial spaces of a target organ is thought to be a critical step of the metastatic cascade (1). In the liver or the bone marrow, this process is facilitated by capillaries that are decorated with discontinuous, fenestrated endothelium (27). However, tumor cell extravasation in the lung is more demanding because of the lung vasculature’s tight endothelial junctions that are in place to prevent fluid leakage and cell passage into the alveolar compartment (28). To overcome this obstacle, lung metastatic tumor cells are equipped with specific factors that promote transendothelial migration such as angiopoietin-like 4, which has been shown to induce endothelial retraction (29). In addition, lung metastasis has been shown to be facilitated by aggregated platelets and fibrin, which together form a blood clot around tumor cells during their prolonged arrest in the lung vasculature (30). The generation of clot around tumor cells is critical as it can protect tumor cells from circulating immune cells, initiate EMT, and provide important biophysical cues to maintain cancer cell stemness (4, 6, 31). Tumor cells adapt to the surrounding clot by producing elongated, axon-like invadopodia, and the capacity to penetrate clot in this specific way correlates with the ability of tumor cells to metastasize to the lungs but not to the liver (5). Here, we show that this specific clot-invasive phenotype is most pronounced in cells from patients with RCC and lung metastasis, indicating that the ability to generate invadopodia in clotted plasma represents a specific adaption for metastasis to the lungs.

Although RCC and STS cell lines produced extensive invadopodia, we found that tumor cells derived from melanoma and pancreatic, breast, and prostate cancers stayed largely rounded after clot embedding, suggesting that interactions with clotted plasma or fibrin are not as important for tumor cells that metastasize more frequently to the liver and bones than to the lungs (25). Notably, the inability to generate invadopodia in clot is not the result of a general inability to invade or metastasize because at least four of the six non-clot interactive tumor cell lines tested have been shown to actively

Figure 5. Integrin αvβ3 promotes fibronectin matrix formation in invadopodia-forming tumor cells. A, analysis of fibronectin matrix formation (green) by confocal microscopy in 24-hour fibrin-embedded 786-O cells after transfection with nonsilencing control siRNA (7860con) or siRNA directed against β1 and β3 integrin (7860-β1/β3) as well as HT1080 and MDA-MB-231 cells. Nuclei are stained with draq5 (blue). Scale bar, 20 μm. B, 786-O cells transfected with control, β3, or FN siRNA were cultured for 48 hours in a fibrin gel and then probed with Alexa Fluor 546 phallolidin to visualize F-actin (red) by confocal microscopy. Fibronectin is shown in green (7860con only). C and D, fibrin-embedded tumor cells were scored for fibronectin matrix formation (C) or stress fiber formation (D) as percentage of total tumor cell count per optical field (50x, stress fiber) using fluorescence microscopy. E, fibrin-embedded 786-O and HT1080 cells were analyzed for invadopodia formation (left, 24 hours) and proliferation (right; 48 hours, control was set to 100%) after treatment with siRNA against fibronectin or nonsilencing control siRNA. F, adhesion of 786-O and HT1080 cells to fibrinogen-coated plates after treatment with siRNA against integrin β3, FN, or nonsilencing control siRNA.
metastasize in mice (PC3, PANC1, MDA-MB-231, A375; refs. 32–35). Moreover, most of the invadopodia-negative cell lines are similar to the invadopodia-positive cells in that they show upregulation of EMT markers and transcription factors in line with previous reports that these cell lines are, in fact, considered to be invasive. One of the major differences between non-clot-invasive and clot-invasive tumor cells, however, is the expression of activated integrin \(v\beta3\), which we found is a prerequisite for adhesion to fibrinogen and invadopodia formation in fibrin. Most invadopodia-negative tumor cells, in contrast, express an inactive form of integrin \(v\beta3\), which correlates with their inability to induce the shape change that is necessary for penetrating a fibrin barrier or navigating narrow interendothelial spaces in the pulmonary vasculature (29).

The proinvasive function of integrin \(v\beta3\) in fibrin-embedded tumor cells is connected to fibronectin, which is significantly expressed in RCC and STS. Fibronectin has been shown to be overexpressed in metastatic tumor cells and tissues, where it promotes invasion, proliferation, and survival through RGD-dependent interaction with integrin (36, 37). Expressed on circulating tumor cells, fibronectin represents a marker for disease progression and treatment resistance (38). Although tumor cells grown on two-dimensional cell culture plastic rarely generate a fibronectin matrix (39), we detected extensive fibronectin matrix assembly around fibrin-embedded RCC and STS cells. Fibronectin matrix formation was strongest in tumor cells from metastatic cancer, whereas cells from localized cancer barely expressed fibronectin at all. This is in line with previous findings that breast tumor cells grown in a 3D environment require fibronectin matrix assembly and stress fiber formation for cell-cycle progression in vitro and metastatic outgrowth in vivo (40). However, while fibronectin-dependent outgrowth of breast cancer cells depends on interactions of fibronectin with \(\beta1\) integrin, we show here that, in fibrin-embedded RCC and STS cells, invadopodia formation, fibronectin matrix assembly, and stress fiber formation are uniquely controlled by integrin \(\alpha\beta3\).

In addition to the capacity to assemble a fibronectin matrix, invadopodia formation correlates closely with the expression of the EMT transcription factor Slug, which also mediated experimental metastasis to the lungs. Slug has been shown to promote invasion and metastasis in a number of cancers and its expression in clinical tumor tissues is frequently associated with poor outcome (13, 41, 42). Specifically, Slug expression has been shown to be enhanced in metastatic RCC, and this correlates with a decrease in progression-free survival (43, 44). Slug expression is commonly induced by TGF-\(\beta\) or Wnt signaling, both of which represent important EMT pathways (13, 14). However, although we detected robust smad2 phosphorylation in RCC and STS cells, we did not find any connection between TGF-\(\beta\) and Slug. Instead, we identified a positive relationship between integrin \(\alpha\beta3\), fibronectin, and Slug expression, indicating that clot invasion in RCC and STS cells is directly controlled by adhesive interactions with the extracellular matrix. This mechanism leads to stress fiber formation, which has been shown to be a prerequisite for the maturation of focal adhesions (45). Importantly, actin-myosin-mediated cellular tension and subsequent focal adhesion formation leads to stabilization and nuclear translocation of \(\beta\)-catenin, a transcription factor critical for Slug expression (46, 47). Moreover, it has been shown that Slug can be induced by the GTPase RhoA, which mediates stress fiber formation in cooperation with integrins and fibronectin (14, 48, 49). Intriguingly, this mechanism could also explain colony formation of tumor cells in fibrin, considering that \(\alpha\beta3\) has been shown to promote proliferation of fibrin-embedded tumor stem cells in a Rho-associated kinase-dependent manner (6).

Based on previous reports that integrin activation is necessary for fibronectin matrix assembly (24), we propose a mechanism where activated integrin \(\alpha\beta3\) generates a fibronectin matrix in fibrin-embedded tumor cells. This leads to stress fiber formation and subsequent upregulation of the EMT transcription factor Slug, which in turn promotes invadopodia formation and lung metastasis. This mechanism is operational in an array of RCC and STS cell lines and appears to be specifically relevant in tumor cells derived from patients with lung metastatic RCC, suggesting that the expression of fibronectin and Slug is indicative for aggressive cancer with poor outcome. Therefore, strategies to inhibit fibronectin matrix formation and Slug expression could...
represent an effective treatment for metastatic RCC as well as STS.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Disha Joshi as well as Michelle Bisceglia and Brandy Greenawalt from the University of Pittsburgh Health Science Tissue Bank for their assistance in obtaining primary tumor cells. The authors also thank Dr. Per Basse and Dr. Simon Watkins (UPCI Cell and Tissue Imaging Facility) for help with confocal microscopy and Dr. Sanford Shattil (University of California San Diego, San Diego, CA) for wowl antibody.

Grant Support
This work was financially supported by National Institutes of Health grants CA134330 (J. Pilch), CA125930 (J.R. Gnarra), and P30CA047904 (UPCI CCSG). This project used the University of Pittsburgh Health Science Tissue Bank, the UPCI Cell and Tissue Imaging Facility, the UPCI Animal Facility, and the UPCI Cytometry Facility, which are supported in part by award P30CA047904. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 1, 2013; revised July 6, 2013; accepted July 26, 2013; published OnlineFirst August 21, 2013.

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