Preventing the Activation or Cycling of the Rap1 GTPase Alters Adhesion and Cytoskeletal Dynamics and Blocks Metastatic Melanoma Cell Extravasation into the Lungs

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Abstract

The Rap1 GTPase is a master regulator of cell adhesion, polarity, and migration. We show that both blocking Rap1 activation and expressing a constitutively active form of Rap1 reduced the ability of B16F1 melanoma cells to extravasate from the microvasculature and form metastatic lesions in the lungs. This correlated with a decreased ability of the tumor cells to undergo transendothelial migration (TEM) in vitro and form dynamic, F-actin–rich pseudopodia that penetrate capillary endothelial walls in vivo. Using multiple tumor cell lines, we show that the inability to form these membrane protrusions, which likely promote TEM and extravasation, can be explained by altered adhesion dynamics and impaired cell polarization that result when Rap1 activation or cycling is perturbed. Thus, targeting Rap1 could be a useful approach for reducing the metastatic dissemination of tumor cells that undergo active TEM. Cancer Res; 70(11); 4590–601. ©2010 AACR.

Introduction

Solid tumor metastasis is a multistep process wherein primary tumor cells become invasive, move through the stroma, penetrate blood or lymphatic vessels, enter the circulation, and traffic to distant sites. To establish secondary tumors at these sites, malignant cells must arrest within and exit from the microvasculature, invade the stroma of the new organ, and proliferate (1–5). Individual steps in this metastatic cascade can proceed by different mechanisms. For example, tumor cell invasion can occur via a "mesenchymal" mode or an "amoeboid" mode (3), distinct types of cell motility that are driven by mutually antagonistic signaling pathways (6). Thus, the design of antimitastatic therapies targeting a particular signaling pathway must take into account both tumor cell–specific and phenotype-specific contexts.

This is especially relevant for the Rap1 GTPase, a central regulator of cell adhesion and motility (7). Extracellular matrix (ECM) ligand binding by integrins leads to increased levels of the active GTP-bound form of Rap1, which, in turn, increases the affinity of integrins for their ligands and promotes the assembly of focal adhesion complexes (7, 8). Additionally, an initial asymmetrical distribution of activated Rap1 promotes cell polarity and migration by remodeling the actin cytoskeleton at the leading edge of the cell (9–13). Thus, Rap1 likely regulates multiple steps in the metastatic cascade. Indeed, altered expression of Rap1, the exchange factors that activate Rap1, or the GTPase-activating proteins (GAP) that convert Rap1 to the inactive GDP-bound state, has been observed in several tumor types, including melanoma (14–19). However, how altered Rap1 activation affects specific aspects of the metastatic cascade is cell type–dependent. For example, activated Rap1 promotes the metastatic invasion of breast, pancreatic, and prostate carcinoma cells but inhibits invasion by osteosarcoma and squamous cell carcinoma cells (14–17, 20, 21).

We now show that Rap1 may be a potential target for impeding the metastatic progression of tumors that exit the vasculature via transendothelial migration (TEM). Modeling the interactions between tumor cells and vascular endothelial cells in vitro showed that both blocking Rap1 activation and enforcing constitutive Rap1 activation altered tumor cell adhesion dynamics and decreased their ability to migrate across endothelial cell monolayers. Importantly, the ability of metastatic B16F1 murine melanoma cells (22, 23) to exit the lung microvasculature and form secondary lesions in vivo was greatly reduced when Rap1 activation was modulated in these ways.
Materials and Methods

Cells

B16F1, A375, MDA-MB-231 [American Type Culture Collection (ATCC)], K1725M1 (Robert Nabi, University of British Columbia, Vancouver, British Columbia, Canada), and bEND.3 cells (K. McNagny, University of British Columbia) were grown in DMEM with 8% FCS. Human umbilical vascular endothelial cells (HUVEC; ATCC) were grown in MCDB131 medium with 20% FCS, 16 units/mL heparin, and 20 μg/mL endothelial cell growth supplement (Sigma-Aldrich).

Reagents

Antibodies used were FLAG (Sigma-Aldrich), Rap1 (Santa Cruz Biotechnology), HUTS4 (BD Pharmingen), paxillin (BD Biosciences), phospho-Y397 focal adhesion kinase (pFAK; Invitrogen), and AlexaFluor555–anti-PECAM-1 (Research Diagnostics). Human fibronectin, laminin, and tumor necrosis factor-α (TNF-α) were from Sigma-Aldrich. Fluorescently labeled phalloidin, AlexaFluor568-dextran, and cell-labeling dyes were from Invitrogen. The pLRM21-IRES-dsFP593 vector and derivatives encoding FLAG-Rap1V12 or FLAG-Rap1GAPII were from Michiyuki Matsuda (Kyoto University, Kyoto, Japan). pEGFP-C1–β1 integrin was from Valerie Weaver (University of California, San Francisco, CA), pEGFP-C1–β1-actin and pEGFP-C1–paxillin were from Robert Nabi. Cellular Lights talin–green fluorescent protein (GFP) was from Invitrogen.

Transfection

To generate stable transfectants, expression vectors plus pMSCVpuro (BD Clontech) were transduced using calcium phosphate and cells were selected with 2 μg/mL puromycin, followed by fluorescence-activated cell sorting for dsFP593 expression. Transient transfections were performed using Lipofectamine 2000 (Invitrogen).

Rap1 activation

Rap1-GTP was precipitated from cell lysates using a glutathione S-transferase–RalGDS fusion protein and detected by immunoblotting with Rap1 antibodies (24).

Immunofluorescence

Cells on ECM-coated substrata were either fixed and stained with antibodies or imaged live in a chamber maintained at 37°C and 5% CO2. Images were acquired using the UPlan ApoChromat 60×/1.35 numerical aperture (NA) objective on an Olympus FV1000 confocal microscope, captured with Olympus Fluoview v1.6 software, and analyzed using ImagePro (Media Cybernetics).

Cell motility

Cells were plated on coverslips coated with fibronectin and a lawn of 1-μm fluorescent microspheres (FluoSpheres, Invitrogen). After 16 hours, the maximum distance from the edge of the cell to the edge of the cleared area was determined using FluoView v1.6 software.

Tumor assays

For subcutaneous tumor growth, 10^5 cells in 0.1 mL DMEM were injected into the hind flanks of C57BL/6 mice. Tumors were analyzed after 14 days. For metastasis assays, 2 x 10^5 cells in 0.1 mL DMEM were injected into the tail vein. After 21 days, the lungs were removed. Visible colonies were photographed and counted.

Arrest and extravasation assays

For competitive arrest experiments, tumor cell populations were differentially labeled with CellTracker Green or CellTracker Far Red dyes and equal numbers (5 x 10^5) were coinjected into the tail veins of C57BL/6 mice. For extravasation assays, labeled cells were coinjected with 5 mg/kg AlexaFluor568-dextran. At the indicated times, lungs were removed, fixed in paraformaldehyde, embedded in OCT, sectioned, stained with 4′,6-diamidino-2-phenylindole (DAPI), and imaged using the UPlan Fluorite 20×/0.7 NA objective of an Olympus FV1000 confocal microscope.

Adhesion under flow

HUVECs were cultured as monolayers on flow chamber slides (IBIDI) and labeled with CellTrace Calcein Red. Tumor cells were labeled with CellTracker Green, mixed with mouse blood, and perfused across the HUVEC monolayer at 2 dynes/cm^2. After 10 minutes, the chamber was flushed and the cells were imaged.

Transendothelial migration

bEND.3 murine microvascular endothelial cells (25) were cultured as monolayers on chamber slides, activated with 50 ng/mL TNF-α, and overlaid with CellTracker Green–labeled tumor cells. After 1 to 24 hours, cells were fixed and stained with rhodamine-phalloidin. Z-stacks were generated by confocal microscopy.

Collagen invasion

Collagen I (2 mg/mL in PBS; BD Biosciences) was polymerized on Transwell filters (Falcon). The lower chamber contained DMEM with 55% FCS and 10 ng/mL transforming growth factor-β (TGF-β; R&D Systems), and 5 x 10^5 CMFDA-labeled B16F1 cells were added to the top chamber. After 20 hours, filters were fixed with paraformaldehyde, embedded in OCT, and sectioned vertically and Z-axis images were acquired.

Adhesion dynamics at tumor cell–endothelial cell interfaces

Tumor cells were added to monolayers of TNF-α–activated bEND.3 cells. After fixation, cells were immunostained with the HUTS4 antibody and imaged by confocal microscopy using the 60×/1.35 NA objective. Fluorescence recovery after photobleaching (FRAP) was performed as described (26, 27) on β1 integrin–GFP–expressing cells. A circular region of interest at the endothelial–tumor cell interface was photobleached using a 405-nm laser (100% intensity, 0.1 s). Fluorescence...
Figure 1. Rap1 regulates focal adhesion formation. A, B16F1 cells were maintained in suspension (−) or plated on fibronectin (FN) or laminin (LAM). Cell lysates were assayed for activated Rap1-GTP and total Rap1. Right, Rap1-GTP levels relative to suspension cells. Columns, mean from three experiments; bars, SD. B, B16F1 cells stably transfected with the empty pIRM21-IRES-dsFP593 vector or derivatives encoding FLAG-Rap1V12 or FLAG-Rap1GAPII (see Supplementary Fig. S1) were plated on fibronectin for 4 h and then immunostained for β1 integrin, paxillin, or pFAK. Representative confocal images of the cell-ECM interface are shown. Scale bar, 20 μm. By setting the threshold for fluorescence detection such that only signals from protein clusters (putative adhesion complexes) were detected, the adhesion area per cell for each protein was determined. Columns, mean for 50 cells from three experiments; bars, SD. C, B16F1 cells that were transiently cotransfected with talin-GFP plus the empty pIRM21-IRES-dsFP593 vector or derivatives encoding FLAG-Rap1V12 or FLAG-Rap1GAPII were plated on fibronectin for 4 h and then imaged. Transfected cells were identified by GFP and dsFP593 (dsRed; inset) fluorescence. Adhesion area per cell for talin-GFP was determined as in B. D, the indicated tumor cell lines were transiently transfected with the indicated vectors and plated on fibronectin for 4 h. The cells were immunostained with anti-FLAG to identify transfected cells and with either a pan-β1 integrin antibody (murine K1735M1 cells) or the HUTS4 antibody, which recognizes only the active form of human β1 integrin (human A375 and MDA-MB-231 cells). Representative confocal images of the cell-ECM interface (see Supplementary Fig. S3) were used to determine the β1 integrin–containing adhesion area per cell, as in B. **, P < 0.01; ***, P < 0.001, compared with control cells.
recovery was imaged until the intensity reached a plateau. The fluorescence signal was normalized to the prebleach intensity, and single exponential fit curves of the data were generated using Prism 4 software (GraphPad).

Intravital microscopy

Mice were anesthetized and the cremaster muscle was exteriorized as described (28). AlexaFluor555-conjugated anti-PECAM-1 (0.2 mg/mL) was injected into the femoral artery along with $5 \times 10^5$ CMFDA dye-labeled B16F1 cells.

Figure 2. Rap1 regulates adhesion and cytoskeletal dynamics, cell polarization, and tumor cell motility. A, vector control, Rap1V12, and Rap1GAPII B16F1 cells expressing paxillin-GFP were plated on fibronectin for 4 h and then imaged continuously for 10 min. Focal adhesions in the first frame (0 min) were pseudocolored green and those in the final frame (10 min) were pseudocolored red before superimposing the two images to identify focal adhesions that were newly formed (red), disassembled (green), or stable (merge = yellow) over the 10-min period. Focal adhesion turnover (% either disassembled or newly assembled) is graphed. Columns, mean for 10 to 15 representative cells from two experiments; bars, SD. B, the indicated cells coexpressing actin-GFP were plated on fibronectin for 4 h and then imaged continuously for 5 min. Top, representative still frames. Scale bars, 20 μm. Bottom, composite images of pseudocolored frames collected at the indicated times. Stable localization of actin-GFP over the 5-min period appears white. The percentage of the total actin-GFP in five equally divided regions of >25 cells from three experiments is graphed. C, vector control, FLAG-Rap1V12–expressing, FLAG-Rap1GAPII–expressing, and Myc-Rap1N17–expressing B16F1 cells were plated for 16 h on fibronectin (2.5 μg/cm²) that was overlaid with fluorescent microspheres. F-actin was visualized with AlexaFluor488-phalloidin (green). Transfected cells were identified by immunostaining for FLAG or Myc (red). Representative confocal images are shown. Scale bar, 100 μm. The maximum distance from the edge of a cell to the edge of the area cleared of fluorescent beads (white arrow) for >50 cells from three experiments is graphed. The horizontal bar is the mean. ***, $P < 0.001$, compared with vector control cells.
The cremaster muscle microvasculature was imaged in real time using an Olympus BX51 upright spinning disc confocal microscope with a ×20/0.95 XLUM Plan Fl objective.

Statistical analysis

P values were calculated using a two-tailed independent two-sample t test.

Results

Modulating Rap1 activation alters focal adhesion formation by tumor cells

Tumor cell motility on two-dimensional substrates requires the activation of ECM-binding integrins and the subsequent formation and turnover of integrin-containing focal adhesion complexes. The Rap1 GTPase is a central regulator of integrin activation, and when murine B16F1 melanoma cells were plated on two-dimensional fibronectin or laminin, the level of active GTP-bound Rap1 increased (Fig. 1A). This led us to hypothesize that Rap1 activation may regulate focal adhesion formation in tumor cells.

To test this, we modulated Rap1 activation in B16F1 cells and other tumor cell lines by expressing either the constitutively active Rap1V12 mutant protein or Rap1GAPII, a Rap-specific GAP that blocks Rap1 activation (Supplementary Fig. S1; refs. 28–31). Imaging the cell-ECM interface such that only adhesive clusters of immunostained proteins were detected showed that Rap1-GTP levels correlated with focal adhesion formation. In both B16F1 (Fig. 1B) and K175SM1 (Fig. 1C) murine melanoma cells, the total area per cell occupied by clustered β1 integrins was increased by Rap1V12 expression and decreased by Rap1GAPII expression. Similarly, using the HUTS4 antibody (32) to detect the active conformation of human β1 integrins in A375 human melanoma cells and MDA-MB-231 human breast carcinoma cells showed that Rap1-GTP levels correlated with the amount of active β1 integrin clustered at the cell-ECM interface (Fig. 1C; Supplementary Fig. S2). The same was true for paxillin and talin, cytosolic proteins that are recruited to focal adhesion complexes, and for the phosphorylated form of FAK (pTAK), an indicator of focal adhesion signaling (Fig. 1B). Consistent with these effects on focal adhesion formation, Rap1V12 expression increased, and Rap1GAPII expression decreased, the adhesiveness of B16F1 cells to rigid substrata (Supplementary Fig. S3). Although Rap1V12 and Rap1GAPII expression had opposite effects on the number of focal adhesions formed, both disrupted cell polarization and focal adhesion dynamics (see below).

Modulating Rap1 activation impairs adhesion dynamics, cytoskeletal dynamics, cell polarization, and cell motility

Cell motility requires the dynamic turnover of focal adhesions and the establishment of a polarized morphology, where integrin signaling drives a dynamic reorganization of the actin cytoskeleton at the leading edge of the cell.
Figure 4. Modulating Rap1 activity impairs melanoma cell extravasation. A, for competitive lung arrest assays, equal numbers of fluorescently labeled vector control B16F1 cells (green) and either Rap1V12- or Rap1GAPII-expressing (red) cells were coinjected i.v. into mice. At the indicated times, lungs were removed, fixed, sectioned, and stained with DAPI to mark all nuclei. Representative sections are shown. The relative number of the different cell populations in 10 to 30 500-μm² sections from four experiments is graphed. Columns, mean; bars, SE. B, to assess tumor cell adhesion to endothelial cells under flow, fluorescently labeled B16F1 cells (green) were perfused across a HUVEC monolayer (red) for 10 min in the presence or absence of a β1 integrin blocking antibody. Representative images of tumor cells adhering to the monolayers are shown. Scale bars, 100 μm. The number of adherent cells per field is graphed. Columns, mean for more than four fields in each of three experiments; bars, SD. ***, P < 0.001, compared with control cells. C, for extravasation assays, fluorescently labeled B16F1 cell populations (green) were injected i.v. together with fluorescent dextran (red) to label the lung microvasculature. After 4 h, lung sections were prepared and imaged as in A. Scale bar, 50 μm. Tumor cells that did not colocalize with the labeled dextran were considered to have extravasated. Yellow, colocalized with dextran within the microvasculature; green, not colocalized, outside the microvasculature. For each cell type, the percentage of imaged cells that had extravasated was determined. Columns, mean for three experiments; bars, SD. **, P < 0.01, compared with control cells. Because Rap1V12- and Rap1GAPII-expressing cells were poorly retained in the lungs, five times as many cells were injected (input) to visualize a sufficient number of cells. D, stably transfected CMFDA-labeled B16F1 cells were added to Transwell chambers in which the filters were coated with collagen I gel. The lower chamber contained FCS and TGF-β as chemoattractants. After 20 h, invasion of the cells into the three-dimensional collagen ECM was imaged. Representative Z-axis images from one of three separate experiments with similar results are shown.
Real-time imaging of paxillin-GFP clustering indicated that vector control B16F1 cells plated on a rigid fibronectin ECM formed new adhesions at their anterior ends and disassembled existing adhesions at their posterior ends (Fig. 2A). In contrast, Rap1V12-expressing cells formed stable adhesions that were localized radially along the cell periphery in a nonpolarized fashion. Rap1GAPII-expressing B16F1 cells formed few focal adhesions, but those that did form were also very stable, with little evidence of a polarized arrangement. Thus, focal adhesion turnover required both Rap1 activation and Rap1 cycling between the active GTP-bound and inactive GDP-bound states.

Modulating Rap1 activation or cycling also prevented B16F1 cells from establishing a polarized morphology with a dynamic actin cytoskeleton. Vector control cells generated a polarized distribution of F-actin (Supplementary Fig. S4A), and real-time imaging of actin-GFP indicated a highly dynamic turnover of actin-GFP filaments at the leading edge (Fig. 2B). In contrast, Rap1V12-expressing cells spread radially and generated nonpolarized parallel arrays of stable actin filaments (Fig. 2B; Supplementary Fig. S4A and B). Rap1GAPII-expressing B16F1 cells formed thin peripheral actin filaments that were also stable and not polarized (Fig. 2B; Supplementary Fig. S4A and B). These effects on actin correlated with a failure to establish a polarized distribution of PIPs (Supplementary Fig. S4B), a lipid that recruits cytoskeletal regulatory proteins. The establishment of cytoskeletal polarity required both Rap1 activation and cycling not only in B16F1 cells but also in K1735M1, A375, and MDA-MB-231 cells (Supplementary Fig. S4C).

The loss of anterior-posterior polarity caused by modulating Rap1 activation, as well as the decreased adhesion and cytoskeletal dynamics, resulted in a significant decrease in cell motility. As assessed using a bead-clearing assay, the movement of B16F1 cells on rigid two-dimensional fibronectin substrata was greatly reduced when Rap1V12 was expressed and when Rap1 activation was blocked by expressing either Rap1GAPII or the dominant-negative Rap1N17 protein (Fig. 2C).

Modulating Rap1 activity inhibits the metastatic extravasation of B16F1 cells from the lung microvasculature

Because altering Rap1 activation had dramatic effects on the in vitro adhesion, polarity, and migration of B16F1 cells, we asked if it interfered with tumorigenesis and metastasis in vivo. Modulating Rap1 activation did not prevent B16F1 cells from forming subcutaneous primary tumors in mice, although Rap1GAPII expression did decrease tumor growth in this context (Fig. 3A). In contrast, both Rap1GAPII and Rap1V12 expression dramatically reduced the ability of B16F1 cells to form metastatic pulmonary lesions after tail vein injection (Fig. 3B).

To identify specific aspects of the metastatic cascade that require Rap1 activation and cycling, vector control B16F1 cells were coinjected into the tail vein with differentially labeled Rap1V12- or Rap1GAPII-expressing B16F1 cells. After 15 minutes, similar numbers of each coinjected cell population were present in the lungs (Fig. 4A). However, after 2 hours, vector control cells vastly outnumbered the Rap1V12- and Rap1GAPII-expressing cells that remained in the lungs (Fig. 4A). Although Rap1GAPII-expressing cells exhibited impaired β1 integrin-mediated adhesion to endothelial cells under flow conditions in vitro (Fig. 4B), a decrease in adhesion to the vascular wall cannot fully account for the dramatic decrease in post-arrest retention in the lungs, as Rap1V12-expressing cells adhered very efficiently to endothelial cells in vitro (Fig. 4B). This suggested that active extravasation might be essential for sustained retention of B16F1 cells in the lung.

To assay extravasation, we injected fluorescently labeled B16F1 cells i.v. and determined their localization in the lungs relative to the capillary microvasculature. Four hours after injection, 45% of the vector control cells in the lung tissue had extravasated and no longer colocalized with the red dextran that was coinjected to mark the microvasculature (Fig. 4C). In contrast, far fewer Rap1V12- and Rap1GAPII-expressing cells moved out of the microvasculature and into the surrounding lung tissue (Fig. 4C). Rap1GAPII-expressing B16F1 cells also exhibited a decreased ability to invade pliable three-dimensional collagen gels in vitro (Fig. 4D). However, Rap1V12-expressing B16F1 cells did invade collagen gels as well as control cells (Fig. 4D), indicating that movement across the endothelial wall is more dependent on Rap1 cycling than the subsequent invasion of the stromal ECM.

Modulating Rap1 activation inhibits TEM by tumor cells

In closed capillary beds such as those of the lungs, tumor cells actively extend membrane processes across the endothelial...
Modulating Rap1 activation alters adhesion dynamics in tumor cells interacting with endothelial cells

TEM by tumor cells requires the dynamic assembly and disassembly of adhesive contacts with the endothelial cells (5). Because modulating Rap1 activation altered the adhesion dynamics of tumor cells on rigid two-dimensional substrata, we determined if this was also the case when tumor cells interact with endothelial cell monolayers. To visualize activated \( \beta_1 \) integrins only in the tumor cells, we seeded A375 and MDA-MB-231 human tumor cells on murine endothelial monolayers and stained them with the HUTS4 antibody that recognizes active human, but not murine, \( \beta_1 \) integrins.

In vector control cells, clusters of active \( \beta_1 \) integrins at the tumor cell–endothelial cell interface were localized in a polarized fashion, predominantly on one side of the cell (Fig. 5C). Rap1GAPII expression significantly decreased the ability of the A375 and MDA-MB-231 human tumor cells to form such adhesive clusters. Moreover, FRAP analyses indicated that the mobility of \( \beta_1 \) integrin–GFP molecules within the adhesions that did form in the Rap1GAPII-expressing cells was increased (Fig. 5D; Supplementary Fig. S5), likely reflecting a reduction in integrin activation and ligand engagement when Rap1 activation is blocked.

Although Rap1V12-expressing tumor cells formed as many active \( \beta_1 \) integrin–containing adhesions as vector control cells, these adhesions were, for the most part, organized radially in a nonpolarized pattern (Fig. 5C). Additionally, FRAP analyses indicated that the mobility of \( \beta_1 \) integrin–GFP molecules within the adhesions of Rap1V12-expressing cells was significantly decreased (Fig. 5D; Supplementary Fig. S6). This decreased rate of integrin exchange may indicate a maturation of the adhesions into stable cytoskeleton-associated complexes. Indeed, disrupting the actin cytoskeleton with latrunculin A greatly increased \( \beta_1 \) integrin mobility within the adhesions of the Rap1V12-expressing tumor cells (Supplementary Fig. S6).

Thus, the reduced ability of Rap1V12-expressing tumor cells to move along, and migrate through, the endothelial monolayer may be due in part to the increased maturity and stability of their adhesive complexes compared with control cells.

Modulating Rap1 activation inhibits the formation of polarized protrusions by tumor cells lodged within capillaries in vitro

To better understand how Rap1 modulation inhibits TEM and extravasation in vivo, we directly imaged the dynamic behavior of B16F1 tumor cells within capillary beds. Because we could not image live cells within the lungs, we injected fluorescently labeled B16F1 cells into the femoral artery and used intravital microscopy to image them within the superficial capillaries of the cremaster muscle, which were marked with fluorescently labeled PECAM-1 antibodies.

We found that the initial arrest of B16F1 cells within the cremaster capillaries seemed to be independent of active cell adhesion. Trypsinizing vector control cells before injection to remove cell surface molecules did not prevent their arrest in the capillaries (Fig. 6A, right). Moreover, Rap1GAPII-expressing B16F1 cells, which adhered poorly to endothelial cells in vitro (Fig. 4B), arrested in the capillaries just as efficiently as nontrypsinized vector controls and the highly adherent Rap1V12-expressing cells (Fig. 6A).

Although it was not technically feasible to image cremaster capillaries long enough to detect completed tumor cell extravasation, real-time imaging of fluorescently labeled or actin-GFP–expressing cells showed that the majority of vector control B16F1 cells that arrested within capillaries formed dynamic, actin-rich pseudopodial protrusions that crossed the endothelial cell wall and entered the interstitium (Fig. 6A–C). The formation of these protrusive pseudopods was significantly reduced in Rap1V12-expressing B16F1 cells and in Rap1GAPII-expressing B16F1 cells (Fig. 6C). Thus, both Rap1 activation and cycling contribute to the formation of dynamic membrane protrusions, which likely facilitate tumor cell TEM and extravasation during metastasis (Fig. 6D).

Discussion

Our results show that modulating Rap1 activation and cycling has distinct effects on different steps in the metastatic cascade. Blocking Rap1 activation in B16F1 mouse melanoma cells resulted in decreased adhesion and migration, as observed previously in human melanoma and human thyroid carcinoma cells (19, 35). This correlated with impaired invasion into three-dimensional collagen gels in vitro, decreased subcutaneous tumor growth, and greatly reduced pulmonary metastasis. Conversely, expressing the constitutively active Rap1V12 protein in B16F1 cells increased adhesion but, surprisingly, also blocked metastatic colonization of the lungs.

The reduced ability of both Rap1GAPII- and Rap1V12-expressing B16F1 cells to form lung metastases seems to be mediated, at least in part, by an impaired ability to exit the lung microvasculature. Previous studies showed that B16F1 cells extend protrusions through the capillary wall and into the subendothelial matrix before extravasation (34). We found that modulating Rap1 activation prevented B16F1 cells from forming such protrusions in vivo. Moreover,
as summarized in Fig. 6D, our in vitro analyses showed that Rap1 activation and cycling were both important for tumor cells that had attached to endothelial cells to form dynamic adhesions and generate actin-rich protrusions that may promote TEM.

Blocking Rap1 activation and expressing constitutively activating Rap1 in tumor cells altered tumor cell–endothelial cell interactions in distinct ways. In Rap1GAPII-expressing tumor cells, reduced TEM was associated with a reduced ability of the cells to form adhesion complexes and spread on the apical surface of the endothelial cells. In contrast, Rap1V12-expressing cells formed robust, stable adhesions but they did not extend polarized protrusions toward the junctions between the endothelial cells and underwent TEM very inefficiently. Although Rap1V12 expression also altered adhesion stability...
and reduced tumor cell migration on rigid two-dimensional ECM substrata, as recently noted (8), this is the first report that this occurs when tumor cells interact with vascular endothelial cells. The highly stable focal adhesions that form in Rap1V12-expressing cells may limit the ability to generate protrusive leading edges that drive tumor cell migration, both on endothelial cells and on rigid substrata.

In contrast to our findings with melanoma cells, expressing constitutively active Rap1 increases the metastasis of prostate carcinoma cells to bone (20). This may reflect differences in the microvasculature of the two target organs. Unlike the closed capillaries of the lungs, the bone marrow sinusoids are mostly open. Thus, prostate carcinoma cells could reach the osteoblastic stroma without actively migrating along, or sending protrusions through, endothelial walls. The increased metastasis of Rap1V12-expressing prostate carcinoma cells may therefore reflect increased invasiveness into the tissue, as opposed to increased TEM. Consistent with this, we found that Rap1V12-expressing B16F1 cells efficiently invaded compliant three-dimensional collagen gels, although they exhibited reduced migration on rigid two-dimensional substrata and reduced TEM across endothelial cell monolayers.

The ability of Rap1V12 expression to inhibit two-dimensional motility but support three-dimensional invasion may relate to the biophysical properties of the substrata (36). Cell attachment to rigid two-dimensional substrata or to the surface of firmly attached endothelial cells may increase cytoskeletal tension within the tumor cell to the point where it augments the ability of Rap1-GTP to stabilize adhesions and impede cell movement; more compliant three-dimensional matrices may not do so. This could be tested directly by altering the stiffness of the collagen matrix, as has been done with breast carcinoma cells (37).

Although several studies concluded that extravasation is not a rate-limiting step in metastatic progression (38–40), our findings suggest that the Rap1-dependent formation of membrane protrusions that promote active extravasation may be a functionally important aspect of the process. Previous attempts to prevent extravasation by targeting matrix metalloproteinases (41, 42), integrins (43), or tetraspanins (5) have been unsuccessful. Our findings suggest that Rap1 could be a novel target for limiting the dissemination of metastatic cells that exit the vasculature via active TEM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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