Activated tumor cell integrin αvβ3 cooperates with platelets to promote extravasation and metastasis from the blood stream

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Running Title:

Integrin activation and platelets support extravasation

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Abstract

Metastasis is the main cause of death in cancer patients and understanding mechanisms that control tumor cell dissemination may lead to improved therapy. Tumor cell adhesion receptors contribute to cancer spreading. We noted earlier that tumor cells expressing the adhesion receptor integrin αvβ3 can carry the molecule in distinct states of activation, and that cells that metastasize from the blood stream express it in a constitutively high affinity form. Here, we analyzed steps of the metastatic cascade *in vivo* and asked when and how the affinity state of integrin αvβ3 confers a critical advantage to disseminating cancer cells. Following tumor cells by real time PCR, non-invasive bioluminescence imaging, intravital microscopy and histology in chick embryos and SCID mice, we identified tumor cell extravasation from the blood stream as a rate-limiting step that is supported by high affinity αvβ3. We found *in vitro* and *in vivo* that successful transendothelial migration depended on cooperation between the tumor cells and platelets involving the high affinity tumor cell integrin and release of platelet granules. Thus, this study identifies activated tumor cell integrin αvβ3 as critical for early steps of metastasis from the blood stream and highlights the high affinity conformation of the receptor and interaction with platelets as potential targets for prevention of metastatic disease.

Introduction

As cancer progresses, tumor cells disseminate from primary tumors to distant sites via the lymphatics or blood stream. Circulating tumor cells attach to the microvasculature of target organs, penetrate the vessel wall, and survive and proliferate within their new tissue microenvironments. Each step can be rate limiting and involve adhesion receptors such as integrins (1). Integrins are heterodimeric transmembrane receptors that recognize extracellular matrix proteins and may exist in high or low affinity states. The affinity can determine ligand recognition and signals that impact cell survival, adhesion, migration, and invasion (2-7). Integrin affinity is regulated inside the cell and controls the adhesive phenotype and ability to bind soluble ligand (8). Integrin activation to high affinity is especially important for leukocytes and platelets, which circulate within the blood stream and can abruptly change their adhesive properties upon stimulation to interact with the vessel wall at sites of vascular injury or inflammation (9-11). Integrin activation can further control cell mobilization from bone marrow and other tissues to enter the circulation and relocate to distant tissue niches (12).

We previously showed that human tumor cells can express the adhesion receptor integrin αvβ3 in distinct states of activation and that tumor cells carrying the high affinity form of the receptor metastasize most efficiently (13). Here, we asked which steps of the metastatic cascade are influenced by integrin activation in tumor cells, and which mechanisms are involved in integrin activation-dependent metastasis. We showed earlier by blood perfusion *in vitro* that expression of αvβ3 in a high affinity state enables tumor cells to interact with platelets and arrest at components of the vessel wall despite shear forces of blood flow (13, 14). Furthermore, we found that activated αvβ3 helps activate metalloproteinase MMP-9 and supports invasive tumor cell migration (15). In the present study, we followed steps of the metastatic cascade *in vivo* and examined if, when, and how expression of activated αvβ3 confers a critical advantage to disseminating tumor cells. We used chick embryos and immune deficient mice to follow tumor cells by real time PCR, non-invasive bioluminescence imaging, intravital microscopy, and histology and analyzed the impact of αvβ3-activation on primary tumor growth, intravasation, tumor cell dissemination, and target organ colonization. The results indicate that expression of high affinity tumor cell αvβ3 is not required for primary tumor growth, but does promote initial steps of target organ colonization from the blood stream to multiple metastatic sites. The mechanism by which activated αvβ3 supports tumor cell access to target tissues depends on platelets which assist tumor cell extravasation.

Materials and Methods

Cell lines, labeling, and *in vivo* model

MDA-MB 435 human tumor cells were from Dr. Janet Price (MD Anderson). Variants of the parental cells lacking αvβ3 and reconstituted with αvβ3WT or αvβ3D723R were previously described (13). Clone E9 cells were isolated by limiting dilution of the parental cells. Parent Combo cells represent a pool of 20 clones, each expressing non-activated αvβ3 based on *in vitro* migration and blood perfusion as detailed below.

For *in vivo* tracking, the tumor cells were stably transduced with red fluorescent protein (*dsRed2)* or firefly luciferase (*Fluc)* using a self-inactivating lentiviral vector (16). For hematogenous metastasis, $1x10^5$ - $1x10^6$ tumor cells were injected into the tail vein of 5- to 8-week old female C.B17/lcTac-*Prkdc scid* mice (Taconic) and metastatic activity followed by non-invasive bioluminescence imaging (IVIS200) (17), fluorescence microscopy of lung whole mounts, or real time PCR of human *alu* sequences in lung extracts using sense primer 5' ACG CCT GTA ATC CCA GCA CTT 3' and antisense primer 5' TCG CCC AGG CTG GAG TGC A 3' (18).

Induction of thrombocytopenia

Thrombocytopenia was induced by i.v. injecting SCID mice with anti-murine GPIbα (CD42b) (Emfret) (3 μg/g bodyweight).

Flow cytometry

Integrin expression and αvβ3 activation were analyzed by flow cytometry (FACS Calibur, Becton Dickinson) with anti αvβ3 mAb VNR1-27.1 (19), or activation-dependent human ligand-mimetic scFv antibodies Bc-12 and Bc-15 (20). ScFv binding was detected with M2 mouse anti-FLAG and anti-mouse-APC using TBS with or without 1mM EDTA, 1mM Ca^{2+}, or 0.1 mM Mn^{2+} as binding and washing buffer.

In vitro migration and arrest during blood flow

Haptotactic migration toward human extracellular matrix proteins was detailed earlier (21). Cancer cell arrest during blood flow was measured as described (13). Briefly, dsRed tagged tumor cells (red fluorescence) were suspended in human blood anticoagulated with 50 nM -Pro-Arg-chloro methyl ketone, spiked with 10 μM mepacrine (green fluorescence) and perfused over collagen I or

subendothelial matrix at a venous wall shear rate of 50 s^{-1} (4 dynes/cm^2). Adhesive events and cell interactions were recorded by fluorescence video microscopy and quantified at 50 predefined positions by image analysis using MetaMorph software.

Dorsal skin fold chamber and intravital microscopy

Tumor cell interaction with the pulmonary microvasculature was observed by intravital microscopy in lung allografts grown in dorsal skin fold chambers on SCID mice as described (22). Briefly, titanium frames were implanted into a dorsal skin fold, transplanted with SCID lung allografts 3 days later, and allowed to vascularize for 14 days. The allografts were superfused with TNFα (50μl at 1μg/ml) 2.5 hours before injecting 2.5×10^6 dsRed tagged tumor cells i.v. together with FITC-dextran (500 kDa) to visualize the vasculature. Adhesive events were observed with an intravital microscope (Leitz Biomed) (22).

Transendothelial migration and platelet preparation

Human pulmonary microvascular endothelial cells were grown for 5 days on transwell filters (8 μm pores) coated with human fibronectin (10μg/ml). Monolayer integrity was measured by transendothelial resistance. Conditioned medium from IMR-90 fibroblasts in the lower chamber was used to attract tumor cells (4×10^4) seeded atop the endothelial layer during a 16 hr migration period. Where indicated, washed human platelets (433×10^3/μl) were added to the tumor cell suspension, with or without stimulation with thrombin receptor activating peptide TRAP-6 (12.5 μM). Non-migrated tumor cells were removed from the filter tops with cotton swaps, filters fixed in 4% paraformaldehyde and transmigrated tumor cells counted by fluorescence microscopy on the filter underside in 5 optical fields.

Results

Tumor cell model for investigating a role of integrin αvβ3 activation in hematogenous metastasis

To identify critical steps within the metastatic cascade affected by the activation state of integrin αvβ3 and to analyze mechanisms involved, we generated a model of tumor cell of variants with a common genetic background and defined levels of αvβ3 expression and activation. We chose MDA-MB 435 human tumor cells because of their aggressive metastatic behavior in immune deficient mice, which makes them suitable for following individual steps of the metastatic cascade (23). The majority of MDA-MB 435 parental cells expresses integrin αvβ3, but this population is heterogeneous and contains a subset that expresses αvβ3 in a constitutively activated functional form, while most cells express the non-activated integrin (13). Our previous results indicate that cells carrying activated αvβ3 are selected as the metastasis forming cells when the parental cells are injected into the blood stream (13). To generate cells with defined states of αvβ3 activation without *in vivo* selection, which may favor additional properties unrelated to αvβ3, we cloned MDA-MB 435 cells by limiting dilution and analyzed 25 individual clones for αvβ3 expression and activation state. Clone E9 was identified as carrying αvβ3 in a constitutively activated form because it bound soluble αvβ3 ligands including the αvβ3 specific ligand-mimetic scFv antibody Bc-15. We previously isolated this antibody from a combinatorial immunoglobulin library and showed that it contains an RGD integrin-binding motif typical for αvβ3 ligands within CDR-H3 (20). Twenty cell clones, each of which expressed αvβ3 but failed to bind Bc-15 and other soluble αvβ3 ligands were pooled and named Parent Combo. These cells uniformly express non-activated αvβ3. Figure 1 summarizes αvβ3 expression and activation in the used cell variants. Bc-15 binding in the presence of calcium indicates that αvβ3 is activated. Binding in the presence of manganese indicates that intrinsically non-activated αvβ3 can be experimentally activated to recognize soluble ligand. Manganese also maximizes intrinsic αvβ3 activation. Based on these criteria, the cells carrying intrinsically activated αvβ3 are clone E9, BCM2, and β3D723R mutant expressing cells. BCM2 were isolated after exposing very low numbers of MDA-MB 435 parental cells to human blood and recovering surviving, proliferating tumor cells. β3D723R cells were generated by reconstituting a β3-lacking subpopulation of MDA-MB 435 (β3^{minus}) with the activating β3 mutant gene. Reconstitution with the β3 wild type gene (β3WT) results in non-activated αvβ3 expression {7809}.

Functional validation of tumor cell integrin αvβ3 activation

Before investigating steps within the metastatic cascade where the activated form of tumor cell integrin αvβ3 may exert its pro-metastatic properties, we verified the functional activation state of the receptor in our cell model. Cells carrying the activating mutant β3D723R were compared against β3^{minus} cells, activated clone E9, and non-activated pool Parent Combo. We analyzed two metastasis related tumor cell functions that are mediated by integrin αvβ3 and which require the activated, high affinity state of the receptor (15, 24). One functions is migration toward a fibrinogen substrate, which is exclusively supported by αvβ3 on these cells (21). The other function is tumor cell attachment to components of the vessel wall during blood flow. This process depends on activated αvβ3 to bind soluble plasma proteins, such as fibrinogen, and cross link the tumor cells with platelets which then supports the arrest event (14). We found that the β3D723R expressing cells as well as clone E9, but not the β3^{minus} and Parent Combo cells, migrated actively toward fibrinogen and this was blocked by an inhibitory anti-β3 antibody (Fig.2A). Consistent with this finding, β3D723R expressing cells and clone E9, but not β3^{minus} and Parent Combo cells were able to arrest on a collagen I matrix during blood flow in a platelet-dependent manner. The interaction of β3D723R cells and clone E9 with platelets during blood flow and the ability of the tumor cells to arrest were inhibited by a function blocking anti-αvβ3 antibody (Fig.2B). Cells expressing β3WT that failed to bind soluble ligand binding as seen in Fig.1 also failed to interact with platelets and arrest during blood flow, confirming that αvβ3 is not activated (Fig.2B). Thus, our cell model contains variants in which the adhesion receptor integrin αvβ3 is expressed either constitutively activated (β3D723R cells and clone E9) or not activated (Parent Combo and β3WT cells). BCM2 cells behaved like β3D723R cells and clone E9, confirming αvβ3 activation (not shown).

Activation of tumor cell integrin αvβ3 promotes initial steps of target organ colonization from the blood stream but does not affect primary tumor growth

To identify steps within the metastatic cascade in which the activation state of tumor cell integrin αvβ3 limits success, we first examined the ability of the cells to form a primary tumor. This was done in chick embryos and in the mammary fat pad of SCID mice. The chick model was chosen because it also allows analysis of tumor cell dissociation from a primary tumor and intravasation (18). After inoculating tumor cells (2x10^6) expressing activated mutant receptor αvβ3D723R versus cells expressing non-activated

αvβ3WT onto the chorioallantoic membrane (CAM) near the allantoic vein of 10 day old chick embryos, tumors were induced by each cell line and grew at similar rates. They also showed similar rates of intravasation, measured by the number of tumor cells in the lower CAM seven days after implantation (578±346 β3D723R, 641±225 β3WT, n=7/group). Quantification was based on real time PCR of the human *alu* sequences as described (18). Similarly, β3D723R expressing cells and those carrying β3WT, as well as β3^{minus} cells grew at comparable rates when injected into the mammary fat pad of SCID mice (Fig.3A). Thus, the expression of αvβ3 and its functional activation state apparently do not limit tumor formation at a primary site or the ability of tumor cells to gain access to the circulation.

We next examined tumor cell colonization of target organs from the blood stream. At this step, expression of constitutively activated αvβ3 provided the tumor cells with a clear advantage. Following tumor cell arrest and expansion in the lungs of SCID mice after tail vein injection by fluorescence microscopy and *alu* PCR indicated that cells expressing activated αvβ3D723R remained in the pulmonary vascular bed and developed metastatic foci more successfully than cells carrying non-activated αvβ3WT. Of $1x10^6$ injected cells, 8.5-$9.5x10^5$ were found in the lungs 3 hours later regardless of αvβ3 activation. For both cell types, this number dropped sharply within the next 12 hours and reached a low point at 24 hours. At this time and during the following 18 days, β3WTexpressing cells were barely detectable in the lungs while β3D723R carrying cells maintained a level of about $1x10^5$ cells at 24 hours and then increased steadily, exceeding $2.5x10^8$ cells compared to only $8x10^5$ β3WT cells on day 35 (Fig.3B). Clearance of tumor cells from lung tissue within the first 24 hours and the early advantage of the β3D723R expressing cells were confirmed by fluorescence microscopy monitoring dsRed tagged cells within the lungs (Fig.3C). Intravital microscopy of lung tissue grafts, grown within dorsal skin fold chambers on SCID mice, allowed to detect the arrest process of β3D723R expressing cells within the pulmonary microvasculature (Fig. 3D). While the arrest events were rare and not suitable for quantification as only small tissue areas could be observed over time, the captured images provide evidence that activated αvβ3 expressing cells can successfully arrest within the pulmonary vascular bed. No arrest events were observed after injection of non-activated β3WT expressing cells. Together, these results indicate that activated integrin αvβ3 endows circulating tumor cells with functional properties that enhance the ability of the cells to colonize the lungs from the blood stream. An initial advantage of tumor cells carrying high affinity αvβ3 translates into enhanced metastatic burden over time.

Integrin αvβ3 activation promotes hematogenous metastasis to multiple sites

We next asked if the advantage conferred to the tumor cells by the high affinity receptor is specific for colonizing the lung, or if αvβ3 activation more generally promotes metastasis from the blood stream. To address this question, we used variants of our cell model that carry intrinsically activated αvβ3. It is not known whether tumor cell subpopulations that express the activated integrin *in vivo* permanently carry the receptor in a high affinity form, or if the activation state is dynamically regulated. Therefore and because of other potential genotypic and phenotypic alterations that might be associated with intrinsic activation of integrin αvβ3, we compared the metastatic activity of tumor cells with intrinsically activated versus non-activated αvβ3 and monitored the distribution of metastatic lesions by non-invasive bioluminescence imaging of SCID mice injected with firefly luciferase (F-*luc*) tagged tumor cells.

E9 cells, cloned *in vitro* from the parent, express intrinsically activated αvβ3 (Fig. 1). When injected into the venous circulation, E9 cells actively produced metastatic lesions in the lungs and other target organs of breast cancer and melanoma metastasis. In contrast, Parent Combo cells, representing a pool of 20 clones with non-activated αvβ3, poorly colonized the lungs and other sites. Figure 4A shows typical organ distribution and quantification of metastatic burden in the lungs comparing clone E9 and Parent Combo cells. The differences in lung colonization were statistically significant when measured 14 days after tail vein injection of $1x10^5$ tumor cells, as well as 34 days after injecting $1x10^6$ tumor cells, to challenge the system. *Ex vivo* imaging of excised target organs on day 56 revealed increased incidence of metastasis to multiple sites for clone E9 expressing the activated integrin (Fig. 4B). This enhancement of overall metastatic activity and potential to colonize most major target organs of breast cancer and melanoma metastasis from the blood stream, was confirmed with BCM-2 cells, a polyclonal derivative of MDA-MB 435 cells that also expresses intrinsically activated αvβ3 (Fig. 1). Tail vein injected BCM-2 cells colonized the lungs, brain, liver, adrenal glands and skeletal bone, as seen by non-invasive bioluminescence imaging and histology (Fig. 4B). Similar results were obtained with β3D723R cells carrying the experimentally activated mutant receptor. These results indicate that expression of high affinity integrin αvβ3 provides blood borne tumor cells with a critical advantage during the colonization of major target organs of metastasis.

Activated tumor cell integrin αvβ3 cooperates with platelets to promote metastasis from the blood stream

To analyze mechanisms by which the expression of tumor cell integrin αvβ3 in a high affinity state supports early steps of metastasis from the blood stream, we followed a key function of the activated integrin that initially lead us to appreciate distinct affinity states of αvβ in tumor cells, namely the ability of the activated receptor to promote tumor cell interaction with platelets (13, 14). Here, we asked if an interaction between tumor cells and platelets could promote not only initial tumor cell arrest within the target organ microvasculature but also subsequent steps of organ colonization. After attaching to the microvascular endothelium of target organs, metastatic tumor cells penetrate the endothelial barrier before invading target tissues. To investigate this step, we analyzed the effect of αvβ3 activation on tumor cell transendothelial migration in the presence or absence of platelets. Comparing cells expressing experimentally (β3D723R) or intrinsically (BCM2) activated αvβ3 against cells carrying the non-activated integrin (β3WT) revealed that either cell type poorly penetrated monolayers of human pulmonary microvascular endothelial cells in the absence of platelets. However, when platelets were present, tumor cells with activated αvβ3 transmigrated very efficiently in contrast to cells with the non-activated integrin. This response was enhanced when platelets were stimulated with thrombin receptor activating peptide (TRAP-6) (Fig.5A). Platelet failed to support tumor cell-transendothelial migration if they had previously released their α-granule contents and were washed before addition to tumor cells and endothelial monolayers (Fig.5A). Platelet releasate alone potentiated tumor cell-induced endothelial retraction in 2D cocultures (not shown). This indicates that tumor cell interaction with platelets, and platelet activation resulting in the release of proteins and stimulants contained in platelet granules, strongly promote tumor cell penetration of the lung microvascular endothelium. Importantly, this cooperation with platelets depended on the activated state of tumor cell integrin αvβ3, as cells expressing the non-activated wild type receptor (Fig.5A) and β3 knock-down cells (not shown) largely failed to penetrate the endothelium regardless of the presence of platelets. TRAP-6, used for platelet stimulation, alone had no effect on tumor cell transendothelial migration (Fig.5A).

To examine if the *in vitro* observed effect of platelets on tumor cell invasion of the lung microvascular endothelium is relevant for hematogenous metastasis *in vivo*, we rendered SCID mice thrombocytopenic and followed the fate of injected tumor cells within a time window of reduced platelet counts. Thrombocytopenia was induced by injecting mice with an antibody against murine platelet receptor

GPIbα. This treatment reduced platelet counts 10-fold from an average of $823 \times 10^3/\mu l$ to $72 \times 10^3/\mu l$ four hours after antibody injection. Counts remained low for 4 days before returning to normal. To examine if platelets support early events of target organ colonization, 5×10^5 tumor cells with activated αvβ3, namely β3D723R cells and BCM2, were injected into the venous circulation 4 hours after treatment with anti-platelet antibody when platelet counts reached the low point. Three days later, within the time of reduced platelet counts, the lungs were examined by histology in serial sections throughout the lung tissue. As shown in Table 1, the number of detectable micrometastases for both tumor cell types was reduced in thrombocytopenic mice. Importantly, it was possible to discern extravasated from intravascular tumor cell nests by analyzing the integrity of basement membranes and the endothelium of the pulmonary microvasculature. Tumor cells clearly surrounded by intact endothelium were classified as intravascular. Tumor cells detected in thrombocytopenic animals largely failed to extravasate, while almost all tumor cells in mice with normal platelet counts were found outside the vasculature (Fig.5B). Furthermore, the majority of tumor cells in mice with normal platelet counts began to proliferate and incorporate BrdU on day 3 post injection, while the percentage of BrdU-positive tumor cells was 3.6-3.9 fold reduced in thrombocytopenic animals (Tab.1). Inhibition of tumor cell extravasation by reducing platelet counts early during target organ colonization from the blood stream translated into reduced numbers and sizes of metastatic foci in the lungs 18 days after tumor cell injection (Fig.5C). Considering that platelet counts remained low only during the first 4 days after tumor cell injection, and then returned to normal allowing micrometastases to develop in the presence of normal platelet counts for the following 14 days, the overall reduction in metastatic burden indicates that platelets indeed played a major role during the initial steps of target organ colonization from the blood stream. Thus, an absence of platelets or the inability of tumor cells to cooperate with them can limit metastatic activity. The finding that activation of tumor cell integrin αvβ3 promotes cooperation with platelets in support tumor cell extravasation as a critical early step of target organ colonization from the blood stream provides a mechanism through which the high affinity form of the integrin promotes metastasis.

Discussion

The metastatic cascade comprises discrete steps in each of which tumor cell musts succeed to establish distant lesions (25). Knowledge about mechanisms supporting individual steps of the dissemination process can help define molecular and functional targets for therapeutic intervention and possibly prevention of metastatic disease.

This study has yielded insight into the role of integrin activation in human tumor metastasis and identified extravasation of circulating tumor cells as a step where the affinity state of tumor cell integrin αvβ3 makes a critical contribution.

The adhesion receptor integrin αvβ3 allows tumor cells to attach and migrate on extracellular matrix proteins including vitronectin, fibronectin, fibrin, and von Willebrand Factor (26). Ligation of the tumor cell receptor can transduce signals that promote survival, proliferation, and expression of genes supporting invasion, growth factor responses and stromal interaction (6, 27). In tumors such as melanoma, glioma, breast and ovarian cancer, αvβ3 appears in invasive margins and distant lesions, and is associated with malignant progression (26, 28-31). Analyzing metastasis promoting tumor cell functions, we noted earlier that tumor cells can express integrin αvβ3 in distinct states of activation and that cells from metastatic lesions carry the integrin in a constitutively high affinity form (13). Importantly, targeting of the activated conformer of αvβ3 on circulating tumor cells with RGD containing ligand mimetic antibodies from cancer patients prevented metastasis from the blood stream (20).

As most prominently shown for platelets and leukocytes, changes in integrin affinity allow the cells to control their adhesive properties within the blood stream and during transit between the circulation and tissues. Prototypic receptors capable of rapid activational changes are platelet integrin αIIbβ3 and leukocyte integrins αMβ2, αLβ2, and α4β1 (4, 8). Furthermore, activation of several other β1 integrins was noted in different cell types associated with their relocation between tissue niches during development, tissue remodeling, and wound healing (7, 32-34). While external signals, agonist stimulation and binding to multivalent ligands can trigger integrin activation from the outside-in (3), constitutive activation as noted for tumor cell αvβ3 in our previous studies likely depends primarily on inside-out signals that lead to constraints in the molecular structure of the α β heterodimer (2).

Crystallographic analyses of αvβ3 (35, 36) showed that large-scale reorientations of the subunit ectodomains are linked to conformational changes in transmembrane domains (4). This involves formation of integrin activation complexes with intracellular integrin binding proteins, such as talin and Rap1-GTP-interacting adaptor molecule RIAM, which results in enhanced receptor affinity without altering receptor valency (8, 37). Recent evidence further suggests that oxidation of redox sensitive cystein residues in integrins can also trigger conformational changes leading to integrin activation and receptor redox modulation (38). This mechanism could be highly relevant in the dynamic control of integrin affinity within microenvironments of activated leukocytes and, perhaps, metastasizing tumor cells that can alter their metabolic properties and production of reactive oxygen species in response to new tissue environments. Our recent proteomic study of bone and brain metastatic cells indicated organ specific metabolic adaptation of the tumor cells with prominent changes in oxidative pathways and redox potential of the cells (17).

To identify steps within the metastatic cascade that are specifically supported by tumor cell integrin αvβ3, among the many other integrins and adhesion receptors expressed by disseminating tumor cells, and to pin-point in which mechanisms the activation state of αvβ3 limits the rate of metastatic progress, we chose a cell model where αvβ3 was identified as important for metastasis and in which proof-of-principle exists for the requirement of αvβ3 activation for the metastatic phenotype (13). We used the MDA-MB 435 cell system because it exemplifies a heterogeneous tumor cell population in which the majority of the cells express non-activated αvβ3, while a small subset carries the high-affinity receptor apparently in a constitutively activated format. Our previous results indicate that this subset is likely responsible for metastasis (13). However, *in vivo* mechanisms providing these cells with a critical advantage during dissemination were unknown. Here we used variants of the MDA-MB 435 model, which share a genetic background and in which the αvβ3 activation state was either controlled experimentally be introducing a β3 mutation that renders the heterodimer consitutively active (β3D723R) (39), or which were selected *in vitro* from the parental cell population and validated for a stable functional activation state of the receptor. In each of these variants, the general integrin expression profile, including that of other αv integrins were unaltered. Thus, this model allowed us to follow the specific contribution of activated αvβ3 to distinct steps of the metastatic cascade, and to identify tumor cell extravasation from the blood stream as a rate-limiting step which requires the high affinity receptor. We found, *in vitro* and *in vivo*, that successful transendothelial migration further

depended on a cooperation between the tumor cells and platelets and involved the release of platelet granules.

Platelet α granules contain a multitude of adhesive ligands, coagulation proteins, pro and anti-angiogenic factors, growth factors, chemokines, cytokines, proteases and bioactive lipid metabolites, many of which directly affect endothelial cells and the integrity of the vascular endothelium. For example, platelet released sphingosine 1-phosphate induces rapid loss of endothelial tight junction proteins ZO-1 and Claudin-18 (40), and lysophosphatidic acid induces vascular permeability (41). Metalloproteinases, and other proteases from platelet α granules, may facilitate extravastation of leukocytes and monocytes, specifically when activated by thrombin within the platelet microenvironment (42, 43). Furthermore, junctional adhesion molecules, such as JAM-C and adhesion receptor GPIbα expressed on platelets, can serve as binding partners for leukocyte integrin αMβ2 and support monocyte and neutrophil extravasation, as well as recruitment of cytotoxic T cells (44-48). Such mechanisms might also be involved in the ability of platelets to support angiogenesis (49, 50).

Our results show that platelets and their released factors can very potently promote tumor cell transendothelial migration, and that platelets are in fact required for extravasation of circulating tumor cells *in vivo*. This platelet-mediated effect, supporting an initial critical step of target organ colonization by circulating tumor cells, depends on the expression of the activated form of tumor cell integrin αvβ3 in our model. It is possible that platelets, activated within the local microenvironment of tumor cells that have arrested within the capillary bed of target organs, promote endothelial permeability and allow tumor cells expressing the activated form of integrin αvβ3 to efficiently use the receptor for penetration of the vessel wall and infiltration of target tissues. This potential mechanism is supported by our previous finding that functional activation of αvβ3 enhances tumor cell migration and promotes activation of metalloproteinases, such as MMP-9 (21). In addition, our *in vitro* perfusion studies indicated that platelets may also critically contribute to tumor cell arrest during blood flow through an interaction mediated by activated tumor cell integrin αvβ3. In its high affinity form, αvβ3 is capable of binding soluble ligands, such as fibrinogen, that can crosslink tumor cells to platelet integrin αIIbβ3 (13, 14). This interaction, resulting in platelet supported tumor cell arrest in the microvasculature, resembles platelet-platelet cohesion during thrombus formation and platelet-leukocyte interaction via platelet receptors and activated leukocyte integrin αMβ2 in inflammation. It is conceivable that platelet supported tumor cell arrest could promote tumor cell interaction with the endothelium in a variety of

capillary beds, potentially with or without help from potential target organ specific endothelial receptors, and assist tumor cells in colonizing multiple tissues in which the tumor cells can thrive. Indeed, our data indicate that tumor cells expressing the activated form of integrin αvβ3 and therefore capable of interacting with platelets, show enhanced metastatic activity in all major target organs examined, and that reduction in platelet numbers lead to a general decrease in metastatic activity from the blood stream. This involves specific adhesive interactions and is not due to unpecific blockage of microvessels by platelet-tumor cell heteroaggregates, because we found no evidence for tumor cell induced thrombus formation in platelet aggregation studies, or thromboembolism by intravital microscopy and histology.

Considering that experimentally induced thrombocytopenia during the initial four days after tumor cell injection strongly inhibited tumor cell extravasation in the mouse model, and with it significantly reduced metastatic burden in the long run, indicates that platelets play a critical role in early steps of the metastatic cascade when tumor cell disseminate via the blood stream. The cooperation between tumor cells and platelets gives tumor cells carrying the activated form of integrin αvβ3 direct, and possibly indirect advantages for target organ colonization. Together, this study identifies the activated form of tumor cell integrin αvβ3 as critical for early steps of metastasis from the blood stream and highlights the high affinity conformation of the receptor as a potential therapeutic target for prevention of metastatic disease.

Figure Legends

Figure 1.

Tumor cell model for integrin αvβ3 activation in tumor metastasis

Flow cytometry analysis of tumor cell variants expressing defined levels and activation states of integrin αvβ3. The cell variants were selected from MDA-MB parental cells *in vitro* or generated by experimental expression of β3 subunit gene variants. Left panels: integrin αvβ3 heterodimer expression (Mab VNR1). Right panels: integrin αvβ3 activation state measured by binding of soluble ligand mimetic scFv antibody Bc-15. This human scFv antibody carries an RGD sequence within CDR-H3 and behaves like a natural ligand of the receptor but has the specificity of an antibody and recognizes αvβ3 only. Lack of scFv binding in the absence of divalent cations (blue lines = no cations) indicates cation dependence. Intrinsic activation of integrin αvβ3 allows scFv binding in the presence of calcium (1mM) (red line = Ca), while low or no binding in calcium indicates non-activated αvβ3 in cells that express the receptor. ScFv binding in the presence manganese (50 μM) (green line = Mn) is maximized, regardless of the intrinsic αvβ3 activation state, as long as the cells are αvβ3 positive. Bold red +/- signs on the right indicate the αvβ3 activation state.

BCM2 cells were isolated by exposing low cell numbers of the parental cell population to human blood and selecting surviving, proliferating tumor cells. These cells express intrinsically activated αvβ3.

Parent Combo cells (PaCo) were selected from the parental cell population by single cell cloning and combining 20 clones each of which express non-activated αvβ3 based on a panel of functional assays, including haptotactic cell migration and blood perfusion as detailed in Figure 2. **E9 cells** represent a single clone isolated by limiting dilution, expressing intrinsically activate αvβ3. **β3 ^{minus} cells** largely lack αvβ3 expression and were selected by exposing the parental cell population to anti-β3 antibody coupled to saporin to kill β3- positive cells. **β3WT cells** are β3^{minus} cells transfected with the β3 wild type subunit gene resulting in the expression of non-activated αvβ3. **β3D723R cells** are β3^{minus} cells transfected with the β3 D723R mutant subunit gene, resulting in the expression of intrinsically activated αvβ3.

Figure 2.

Functional validation of tumor cell integrin αvβ3 activation

A, Haptotactic migration toward immobilized fibrinogen requires expression of integrin αvβ3 in an activated functional state. MDA-MB 435 cells selected for lack of β3 expression (β3 minus) failed to

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migrate in contrast to their counterparts reconstituted with the constitutively activating β3mutant D723R (p<0.002). Migration of β3 mutant D723R expressing cells is strongly inhibited by blocking anti-β3 antibody (mab M21-3 at 50 µg/ml) (p<0.002). E9 cells, a single clone isolated from parental MDA-MB 435 cells by limiting dilution, express the same amount of αvβ3 as Parent Combo cells, a pool of 20 clones expressing the non-activated integrin, but in contrast to Parent Combo cells, E9 cells express αvβ3 in an intrinsically activated form and migrated actively toward fibrinogen (p<0.001) (16 hr migration at 37C toward human fibrinogen immobilized at the underside of transwell insert filters with 8 µm pores).

B, <u>Left Panel</u>: The ability of tumor cells to attach during blood flow to matrix components of the vessel wall depends upon and indicates the activated state of tumor cell integrin αvβ3. MDA-MB 435 β3 minus, β3D723R, E9, and Parent Combo cells were suspended in whole human blood and perfused over a collagen I matrix at a venous wall shear rate of 2 dynes/cm^2 in a parallel plate flow chamber. Tumor cells expressing activated αvβ3 use the receptor to interact with platelets and, as a result, arrest at the matrix in the presence of shear generated by blood flow. <u>Middle Panel</u>: In β3D723R expressing cells and clone E9, the ability to arrest during blood flow is mediated by αvβ3, as this function is blocked by anti αvβ3 antibody (mab VNR1-27.1 at 50 µg/ml). <u>Right Panel:</u> Typical images, captured at predefined positions, during flow of blood containing either MDA-MB 435 β3D723R (left) or β3WT (right) expressing cells. The top images, acquired with filter settings for green fluorescence (top images) show arrested blood cells and thrombus forming platelets. The bottom images were captured at the same positions as their corresponding top images but with filters detecting red fluorescence identify the arrested tumor cells. Arrested tumor cells were quantified at 50 predefined positions during ongoing perfusion after same number of tumor cells for each cell type were allowed to pass through the chamber. Statistical significance was assessed by paired, one-tailed *t*-tests.

Figure 3.

Activation of tumor cell integrin αvβ3 promotes initial steps of target organ colonization from the blood stream but is not required for primary tumor growth

A, Tumor cell growth within the mammary fat pad as a primary site does not depend on integrin αvβ3 expression or the activation state of the receptor. MDA-MB 435 cells selected for lack of β3 expression (β3⁻), reconstituted with β3 wild type (β3WT), or with the constitutively activating mutant β3 (β3D723R) grew at similar rates after injecting 1x10^6 cells into the mammary fat pad of SCID mice

(n=8). Tumor sizes were measured with calipers by the longest and shortest diameter and calculating the volume as $(a^2xb)/2$.

B, Quantification of tumor cells in SCID mouse lungs at varying time points after tail vein injection of $1x10^6$ dsRed tagged MDA-MB 435 cells expressing either constitutively activated $\alpha v\beta 3D723R$ or non-activated $\alpha v\beta 3WT$. Tumor cells were quantified at the indicted time points by real time PCR of human *alu* sequences within the lung tissue. The initial signal at 3h post injection was nearly identical for both cell types and reflects tumor cells within the pulmonary microcirculation, most of which are cleared during the first day. Bars indicate numbers of lung associated tumor cells (n=5 mice), shown as percent (± SEM) of the initial tumor cell number at 3 h post injection. Cell numbers are based on PCR standard curves, obtained from lung homogenates spiked with known tumor cell numbers.

C, Lung whole mounts 3 to 12 hrs after tail vein injection of dsRed tagged tumor cells as in B, visualizing the tumor cells by fluorescence microscopy (10x). Representative fields are shown.

D, Arrest of $\beta 3D723R$ expressing cells within the pulmonary microvasculature observed by intravital microscopy. Lung allografts were grown in dorsal skin fold chambers on SCID mice, allowed to vascularize for 14 days before superfusion with TNFα (50μl at 1μg/ml) 2.5 hours prior to i.v. injection of $2.5x10^6$ dsRed tagged tumor cells together with FITC-dextran (500 kDa) to visualize the vasculature. Circulation and adhesive events were recorded by intravital video microscopy (Leitz Biomed). Bar, 50 μm.

Figure 4.
Integrin $\alpha v\beta 3$ activation promotes hematogenous metastasis to multiple target organs
A. <u>Left panels:</u> Metastatic activity of MDA-MB 435 clone E9 expressing intrinsically activated integrin $\alpha v\beta 3$ compared to 20-clone pool Parent Combo expressing non-activated $\alpha v\beta 3$. Non-invasive bioluminescence imaging of SCID mice 14 days after tail vein injection of $1x10^5$ F-luc tagged tumor cells. <u>Right panels:</u> Quantification of metastatic burden in the lung region by non-invasive bioluminescence imaging 14 days after injecting $1x10^5$ tumor cells (Top), or by real time PCR 34 days after injecting $1x10^6$ tumor cells to challenge the system and following the tumor cells by human a*lu* sequences (Bottom). Each data point in the top panel represents signal from one mouse (n=8), the vertical line indicates the median signal in each group. Bars in the lower panel show average values (+/- STDEV) for each group (n=8). Clone E9 caused significantly higher metastatic lung burden than clone pool Parent Combo (p=0.002 by bioluminescence imaging on day 14 after $1x10^5$ injected tumor cells ;

p= 0.05 by *alu* PCR 34 days after $1x10^6$ injected tumor cells). Statistical significance was assessed by paired, one-tailed *t*-tests.

B. Comparison of target organ colonization by tumor cells expressing activated versus non-activated αvβ3. Incidence of tumor cell burden in the lungs, brain, liver, adrenal glands and bone (hind legs) 56 days after tail vein injection of $1x10^5$ clone E9 versus clone pool Parent Combo. Organ associated metastatic burden was detected in excised organs by ex vivo bioluminescence imaging, n= 8 mice per group. The presence of metastatic lesions was verified by histology (right panels). Brain lesion H&E staining, Bar 80μm. Liver lesion, top panel identifies tumor lesions by immunohistochemistry with anti human CD44 (Mab 29.7) (dark blue), bottom panel shows a neighboring section stained with H&E and lesions marked with blue asterisks, Bar 200 μm. Adrenal gland section shown by H&E staining, lesions marked with blue asterisks, Bar 150 μm. Mouse images: Multiple target organ colonization validated with polyclonal cell population BCM2 which express activated integrin αvβ3. Non-invasive bioluminescence imaging of metastasis development in distinct target organs 56 days after tail vein injection $2.5x10^5$ tumor cells. Three representative mice are shown (ventral and dorsal). Metastatic burden in the lungs, liver, brain, spine, and adrenal gland was confirmed by bioluminescence imaging of the excised organs and by histology as above.

Figure 5.
Activated integrin αvβ3 promotes tumor cell cooperation with platelets in support of extavasation and metastasis from the blood stream

A. Platelets and activated tumor cell integrin αvβ3 together promote tumor cell transendothelial migration in vitro. In the presence of platelets, MDA-MB 435 cell variants with activated mutant αvβ3D723R, or expressing intrinsically activated αvβ3 (BCM2), penetrated monolayers of human lung microvascular endothelial cells more efficiently than tumor cells with non-activated αvβ3WT (p=0.03). Experimental platelet activation with TRAP-6 (thrombin receptor activating peptide-6, a PAR1 agonist) before addition to the tumor cells, slightly enhances the stimulatory effect of platelets on tumor cell transendothelial migration (p=0.13 for βD723R, p=0.21 for BCM2). TRAP-6 alone, or exhausted platelets stimulated with TRAP-6, allowed to release their α granules and washed before addition to tumor cells, did not enhance tumor cell transendothelial migration.

B. Experimental thrombocytopenia prevents tumor cell extravasation in vivo and inhibits metastasis. SCID mice were injected with a polyclonal antibody against mouse platelet glycoporotein GPIbα (3μg/g

bodyweight) to induce temporary thrombocytopenia. After 4 hrs, platelet count were reduced from an average of $823 \times 10^3/\mu l$ to an average of $72 \times 10^3/\mu l$ and remained low for 4 days before returning to normal. 4 hours after induction of thrombocytopenia, 5×10^5 β3D723R expressing cells or BCM2, both expressing activated integrin αvβ3, were injected into the tail vein. Representative microscopic images of lung sections show tumor cells 3 days after injection into mice with normal platelet counts (top) or 10-fold reduced platelet counts (bottom). With normal platelet counts, multiple tumor cell clusters (green arrows) are seen within the lung tissue outside of blood vessels. With reduced platelet counts, few small tumor cell clusters are seen and almost always found inside blood vessels (green arrow). Bars 100 µm. Quantification of in vivo tumor cell extravasation on Day 3 is summarized in Table 1.

C. Experimental thrombocytopenia reduces metastasis from the blood stream. SCID mice were injected with anti platelet glycoprotein GPIbα as above or PBS for control (n=6). Four hours later the platelet count had dropped from an average of $775 \times 10^3/\mu l$ to an average of $65 \times 10^3/\mu l$ in the antibody treated mice. At this time point, 1×10^5 sdRed tagged BCM2 tumor cells were injected into the tail vein and lung metastases counted microscopically in whole mounts of each lung lobe 18 days later. Bars denote the number of metastatic foci per lung ± SEM, counted regardless of size. Images below show dsRed tumor cell lesions in lung whole mounts representative for each experimental group. Mice with normal platelet counts had significantly more metastases (p<0.05) than mice with thrombocytopenia. Generally, lesions in control mice were also considerably larger than lesions in anti-platelet treated mice. Differences in metastatic burden were confirmed by real time PCR of human *alu* sequences within the lung tissue (not shown). Statistical significance was assessed by paired, one-tailed *t*-tests.

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Figure 1

αvβ3 expression   αvβ3 activation

BCM2   BCM2
                                     no   Ca   Mn   +

PaCo   PaCo
                                     Ca   Mn   −
                                     no

E9   E9
                                     no   Ca   Mn   +

β3⁻   Ca   β3⁻
                                     no   Mn

β3WT   β3WT
                                     Ca   Mn   −
                                     no

β3D723R   β3D723R
                                     Ca   Mn   +
                                     no

Figure 2

A αvβ3 activation dependent migration

p < 0.002
p < 0.002
p < 0.001

Migrated tumor cells

350
300
250
200
150
100
50
0

β3 D723R | β3 D723R + anti β3 | β3 minus | E9 | PaCombo

B Platelet mediated tumor cell arrest during blood flow

No inhibitor

p < 0.001
p < 0.001

Adherent tumor cells

β3 D723R | β3 minus | E9 | PaCombo

Anti-αvβ3

600
500
400
300
200
100
0

β3 D723R | E9

Typical images of platelet and tumor cell arrest during blood flow

All Cells + Thrombi

Tumor Cells

Top and Bottom at same position

β3 D723R | β3 WT

Figure 3

A — Tumor volume (mm³) vs Days post implantation; β3WT, β3⁻, β3D723R; n = 8 each group

B — Tumor cells in the lungs, % cell of signal seen at 3h vs Time after injection; αvβ3$_{D723R}$, αvβ3$_{WT}$; n=5 each group

C — β3$_{D723R}$ and β3$_{WT}$ at 3h, 24h, 6d, 12d

D — arrest of β3$_{D723R}$; Before, After
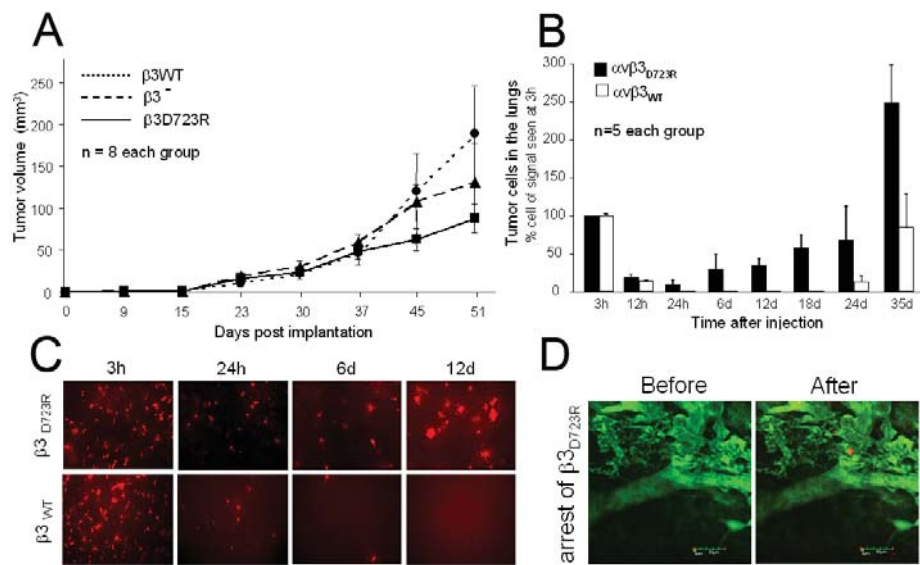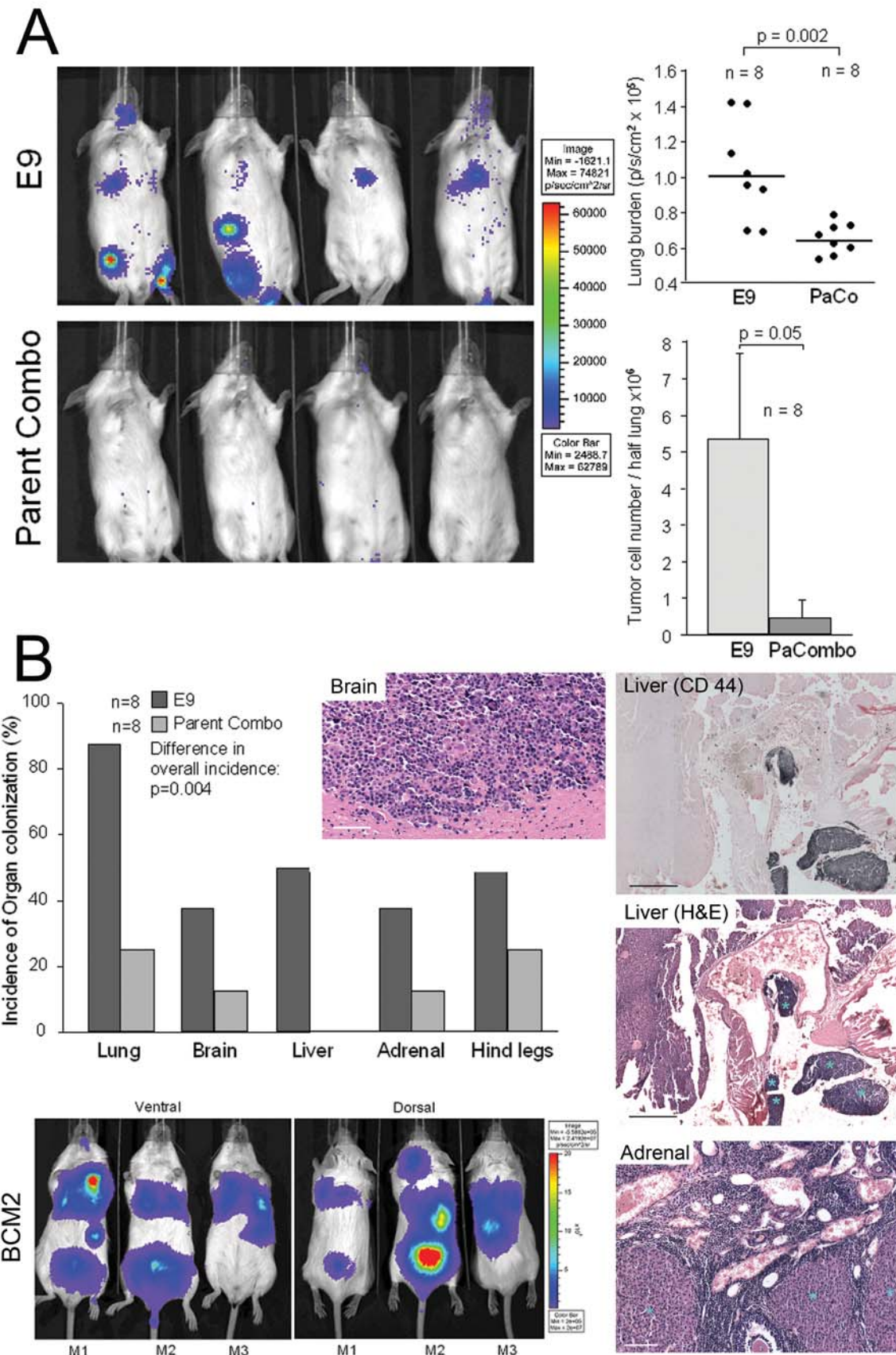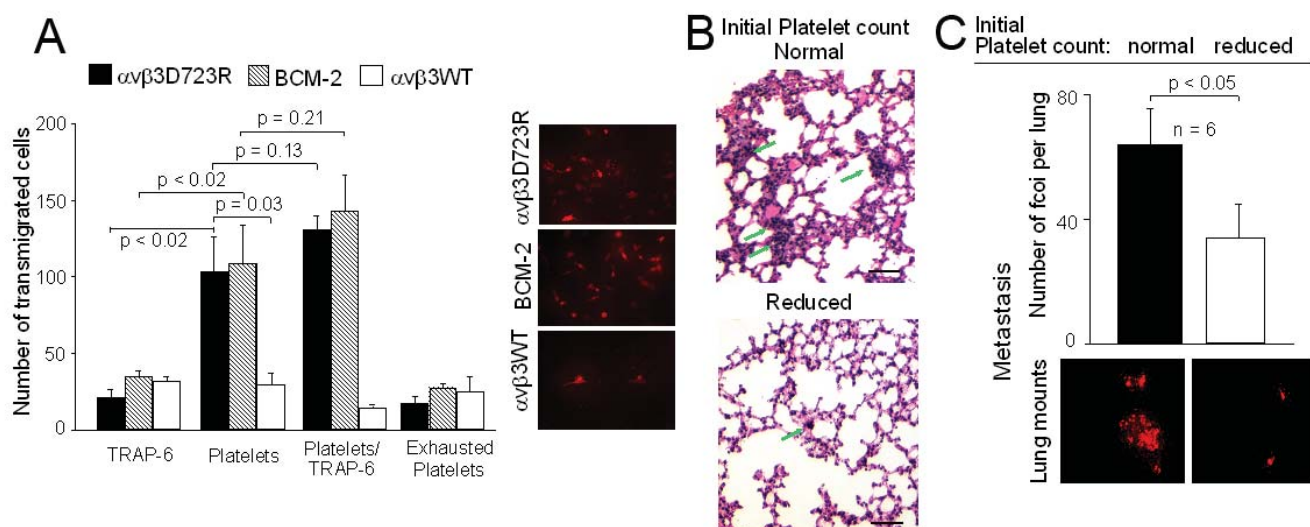
Figure 4

A

B

Figure 5

Tumor cell type	Platelet count	Measured lung area (mm^2)	Number of metastases in measured area	Number of metastases / mm^2	% extravasated	% BrdU positive
β3D723R	normal	85.3	35	0.410	100	68.6
		140.2	83	0.592	95	62.4
β3D723R	reduced	109.6	9	0.082	0	15.5
		126.2	22	0.174	0	18.6
BCM2	normal	84.4	52	0.616	92	67.2
		88.7	38	0.428	100	63.0
BCM2	reduced	84.3	14	0.166	7	12.2
		86.7	18	0.207	0	14.4

Table 1. Effect of platelets on tumor cell extravasation and early metastasis in vivo.

Morphometric measurements of mouse lung sections 3 days after i.v. injection of MDA-MB 435 β3D723R or BCM2 cells. Each cell type was injected into mice with normal or 10-fold reduced platelet counts (n=2/condition). Data for each mouse indicate the analyzed lung area, numbers of metastases found (lesion count regardless of size), number of metastases/area, percentage of lesions found intravascular (intact vascular endothelium/basement membrane), or near blood vessels with broken vascular basement membrane indicating extravasation. Also included is the percentage of lesions showing BrdU uptake (24 h *in vivo* pulse) indicating proliferative activity, regardless of lesion size. Tumor cell lesions in mice with normal platelet counts were generally considerably larger and had higher proliferative activity than lesions in thrombocytopenic mice.