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Metastatic Disease: Interactions Between Tumor Cells and Host Environment During Cancer Cell Spread

Jennifer M. MacLean, *University of Western Ontario*

Supervisor: Drs Ann F Chambers, *The University of Western Ontario*
Joint Supervisor: Ian C MacDonald, *The University of Western Ontario*
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METASTATIC DISEASE: INTERACTIONS BETWEEN TUMOR CELLS AND HOST ENVIRONMENT DURING CANCER CELL SPREAD

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Jennifer M MacLean

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THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

CERTIFICATE OF EXAMINATION

Supervisor

Dr. Ann F. Chambers

Dr. Ian C. MacDonald

Supervisory Committee

Dr. Alan B. Tuck

Dr. Chris G. Ellis

Dr. Frank Beier

Examiners

Dr. Janusz Rak

Dr. Vincent Morris

Dr. Paula Foster

Dr. John Lewis

The thesis by

Jennifer Margaret MacLean

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Abstract

Tumor and metastasis formation are not cell autonomous phenomena, but rather an evolution of disease within and responding to the host environment. Metastatic spread from a primary tumor occurs as a result of a complex interplay between tumor cells and the host, wherein tumor cells must escape the primary tumor, enter the host vasculature, travel to and arrest in a distant tissue and survive and grow in that new organ. It is known that cells that progress through these stages must both escape and exploit host systems, yet the mechanisms used are not fully understood. Therefore, the goal of this work was to investigate the interactions between tumor cells and the host and to determine the role that host systems play in supporting tumor development and progression. Specifically, the interaction between hemostatic factors and metastatic cells was evaluated, and the effect of a primary tumor in influencing this interaction was investigated using a murine melanoma cell line. It was determined that tumor cells are capable of exploiting host hemostatic factors to increase lung metastasis, and stabilization of this interaction increased metastasis. Intriguingly, the presence of a primary tumor depleted circulating platelets leading to impaired hemostasis at the time of metastatic cell introduction. This led to reduced interaction between tumor cells and host hemostatic factors and decreased metastasis. To determine if this effect of a primary tumor was unique to melanoma, a human breast adenocarcinoma cell line was also used. In agreement with murine melanoma data, it was found that the presence of a breast primary tumor also reduced the development of lung metastases. The ability of metastatic cells to exploit host hemostatic factors and the identification of global modulation of this interaction by a primary tumor indicates that the interaction between primary tumor, host, and distant metastatic cells is complex and multi-faceted. Full understanding of the interplay between a primary tumor, the host and metastases will be essential to the development of strategies to inhibit metastatic progression, either before or after the surgical resection of the primary tumor.

Keywords

Pre-clinical, Metastasis, Hemostasis, Melanoma, Breast Cancer, Concomitant tumor resistance

Co-Authorship Statement

The following thesis contains material from previously a published manuscript from Clinical and Experimental Metastasis, a submitted manuscript, and a text chapter written for inclusion in Experimental and Clinical Metastasis: A Comprehensive Review. Copyright permissions as required for thesis publication are provided in Appendix A.

Chapter 1 contains sections from a chapter included in "Experimental and Clinical Metastasis: A Comprehensive Review - In Press (Springer)", entitled "Interactions of Normal Tissues and Systems with Metastatic Cells: Impact on Location, Survival and Growth", written with the assistance of AF Chambers.

Chapter 2 had been published as "Effect of anti-fibrinolytic therapy on experimental melanoma metastasis" by JM Kirstein, KC Graham, LT MacKenzie, DE Johnston, LJ Martin, AB Tuck, IC MacDonald, and AF Chambers in Clinical and Experimental Metastasis 2009;26(2):121-31.

I designed and carried out the experiments described in this publication. The B16F10-LacZ cell line was generated by KC Graham, LT MacKenzie provided assistance with animal injections, DE Johnston provided histological sectioning and staining, and LJ Martin performed confocal quantification as part of an undergraduate research project. IC MacDonald and AF Chambers provided supervision and assistance editing the manuscript.

Chapter 3 has been submitted for review to the International Journal of Cancer as "Primary melanoma tumor decreases metastasis through alterations in systemic hemostasis" by JM Kirstein, MN Hague, PM McGowan, AB Tuck, and AF Chambers.

I designed and carried out the experiments described in this publication. MN Hague, PM McGowan and I worked as a co-ordinated team during the extensive animal experiments. MN Hague provided expert assistance with animal injections, PM McGowan performed the platelet isolation and I prepared tumor cells for injection. MN Hague also performed confocal analysis. Assistance with interpretation of murine spleen data, was provided by

I Welch at Animal Care and Veterinary Services at the University of Western Ontario. AB Tuck assisted with histological analysis and he and AF Chambers provided supervision and assistance editing the manuscript.

Chapter 4 outlines ongoing work within our laboratory that will be included with Chapter 3 for submission. I designed and carried out the experiments described here and received assistance from MN Hague with animal injections. Confocal images were taken by CD MacMillan, flow cytometry was performed in collaboration between C Simedrea and DW Dales and BD Hedley, and histological staining was performed by CO Postenka. AB Tuck assisted with histological analysis and he and AF Chambers provided supervision and assistance editing the chapter.

Throughout the thesis, figure formatting and image processing assistance was provided by KA MacLean.

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Abbreviation List

231LN – metastatic variant of the MDA-MB-231 cell line

3D – 3-dimensional

AT – antithrombin

BMDC – bone marrow derived cells

BSGC – buffered saline glucose citrate

CAF – carcinoma associated fibroblast

CAM – chorioallantoic membrane

CMFDA – Cell tracker Green (5-chloromethylfluorescein diacetate)

CSF-1 – colony stimulating Factor -1

EACA – ε-amino caprioic acid

ECM – extracellular matrix

EDTA – ethylenediaminetetraacetic acid

EGF – epidermal growth factor

EMT – epithelial-mesenchymal transition

FBS – fetal bovine serum

FN – fibronectin

H&E – hematoxylin and eosin

HBSS – Hanks balanced salt solution

HPC – hematapoietic progenitor cells

KIU – kallikrein inhibiting units

LLC – lewis lung carcinoma

LMWH – low molecular weight heparin

MFP – mammary fat pad

MMP – matrix metalloprotease

MV – microvesicles

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

NK – natural killer

NO – nitric oxide

OPN – osteopontin

PAF – platelet-activating factor

PAR – protease activated receptor

PAI – plasminogen activator inhibitor

PDGF – platelet derived growth factor

PL – phospholipid

PlGF – placental growth factor

PR – platelet rich

PVDF – polyvinylidene fluoride

SCC – squamous cell carcinoma

SDF-1 – stromal derived factor-1

TAM – tumor associated macrophage

TBS-T – tris-buffered saline with 0.1% Tween

TF – tissue factor

TFPI-1 – tissue factor pathway inhibitor -1

TGFβ – tumor growth factor- β

TNFα – tumor necrosis factor α

tPA – tissue type plasminogen activator

TSP – thrombospondin

TXA – tranexamic acid

UFH – unfractionated heparin

uPA – urokinase type plasminogen activator

uPAR – urokinase type plasminogen activator receptor

VEGF – vascular endothelial growth factor

VEGFR – vascular endothelial growth factor receptor

VTE – venous thromboembolism

VWF – von Willebrand factor

Chapter 1

1 Introduction

Cancer was responsible for 7.9 million deaths in 2007, accounting for approximately 13% of all deaths worldwide. This number is expected to climb to over 11 million deaths by 2030[1]. Importantly, primary tumor formation is not generally responsible for this high level of mortality. Rather, the spread of tumor cells throughout the body in a process known as metastasis leads to the majority of cancer-related deaths[2]. In this thesis I will focus on how tumor cells are capable of exploiting host cells, growth factors, pathways and systems during each of the key steps in metastasis.

1.1 Cancer is a disease of cell and host

Tumor formation is not a cell autonomous phenomenon, but rather an evolution of disease within and responding to the host environment. In particular, metastatic spread from a primary tumor occurs as a result of a complex interplay between tumor cells and the host. In order to form successful metastases, tumor cells must escape the primary tumor, enter the host vasculature, travel to and arrest in a distant tissue and survive and grow in that new organ[2]. Cells that progress through these stages must both escape and exploit host systems.

As tumor cells acquire a metastatic phenotype, they do so through interacting with and manipulating host responses[3-5]. The tissue microenvironment is significantly altered by the presence of a primary tumor, with changes in stromal cell composition and the presence of infiltrating immune cells. The individual components are specific to tumor type, but the net result is a cycle of mutual stimulation of host and tumor tissue, leading to increased tumor growth and aggressive behavior.

Many components and host systems have been identified to play a role in tumor progression and metastasis, but the interaction between tumor cells and components of the host hemostatic system plays a particularly important and pervasive role in metastasis.

1.1.1 Hemostasis

The human circulatory system has evolved to react to vascular injury in an explosive manner to prevent excessive blood loss. This rapid response exists in a delicate balance with tight controls and regulation; initiation of coagulation is followed closely by activation of fibrinolysis which enables the hemostatic system to stem excessive blood loss, without giving rise to thrombosis[6]. An imbalance or defect in any component of the hemostatic system can lead to a clinical disorder, such as hemophilia upon the loss of Factor VIII[7]. The major components and tightly regulated pathways and the interplay between coagulation, fibrinolysis, and tumor progression will be discussed here.

1.1.2 Vascular endothelium in hemostasis

The vasculature is not just a passive conduit for blood circulation; rather the endothelial cells lining blood vessels are active participants in hemostasis[6]. For example, endothelial cells secrete basement membrane and extracellular matrix (ECM) components such as collagen, fibronectin (FN), laminin, vitronectin and von Willebrand Factor (VWF), all of which are pro-thrombotic if allowed to contact blood components[6]. To protect these proteins from spontaneous contact with the blood, endothelial cells secrete the anti-thrombotic molecules thrombomodulin and heparin sulphate on their surface. Upon stimulation by enzymes like thrombin or under hypoxic or shear stress, leads to tissue factor (TF) and VWF presentation on their cell surface and alters integrin expression. This facilitates platelet adhesion and increased FN, collagen and laminin binding. Thus, the endothelium potentiates a pro-coagulant response. Additionally, damage to the vessel wall causes blood exposure to sub-endothelial proteins which stimulate formation of platelet aggregates and thrombi[6].

1.1.3 Platelets

Platelets are small (2.5 x 0.5 um) anuclear cytoplasmic bodies which are formed by fragmentation of megakaryocytes in the bone marrow[8]. As a major component of the hemostatic system, 150 - 400 $x10^9$ platelets per litre of blood normally circulate within the human vasculature, but do not adhere to the vascular wall unless stimulated to do so[8, 9]. In response to vascular injury, platelets adhere to the exposed sub-endothelium and

become activated. This activation causes platelets to change from a flat discoid shape to a more spherical form with extensive pseudopodia and causes the release of proteins from α-granules[8]. Activation also causes extensive changes on the platelet membrane with increased presentation of activated GPIIb/IIIa ($\alpha_{IIb}\beta_3$), which allows for binding to fibrinogen, VWF, FN and vitronectin. Release of fibrinogen from α-granules contributes to stabilization of the fibrin clot, as $\alpha_{IIb}\beta_3$ on the platelet surface mediates binding to fibrinogen as well as FN and VWF.

1.1.4 Coagulation Cascade

Two separate proteolytic cascades are responsible for initial activation of coagulation – the intrinsic and extrinsic pathways[6]. The intrinsic pathway is activated by tissue damage when blood comes into contact with sub-endothelial tissues. It is responsible for the initial reaction to tissue damage, but is slower than the extrinsic pathway to activate the key protease, thrombin. The extrinsic pathway provides a rapid response to coagulation stimuli and functions mainly to augment the activity of the intrinsic pathway. Both the intrinsic and extrinsic pathways lead to the common pathway, which results in thrombin formation after activation of Factor X. The central component to cascade progression involves binding of the co-factor TF to phospholipid (PL, intrinsic) or Factor VIIa (extrinsic) to activate Factor IX and Factor X respectively.[6] All of the serine proteases involved in the coagulation cascade circulate as inactive zymogens which prevents spontaneous clot formation, while still enabling a rapid response to vascular injury. Figure 1.1 illustrates the progressive nature of the clotting cascade, and outlines the individual protein-cofactor interactions required. In general, exposure of the subendothelium triggers the intrinsic cascade leading to pro-thrombin conversion to activated thrombin[6]. Thrombin cleaves fibrinogen to the self-polymerizing protein fibrin to initiate the second phase of clot formation, and also contributes to clot expansion through increasing activation of Factor VIII, X and XI. Formation of a stable fibrin clot occurs through thrombin-mediated cleavage of fibrinogen to fibrin, and the subsequent crosslinking by Factor XIIIa. This fibrin mesh binds platelets together and increases attachment of the thrombus to the damaged vessel wall and binds to the platelet receptor $\alpha_{IIb}\beta_3$. Molecular bridges between fibrin and the plasma proteins FN and

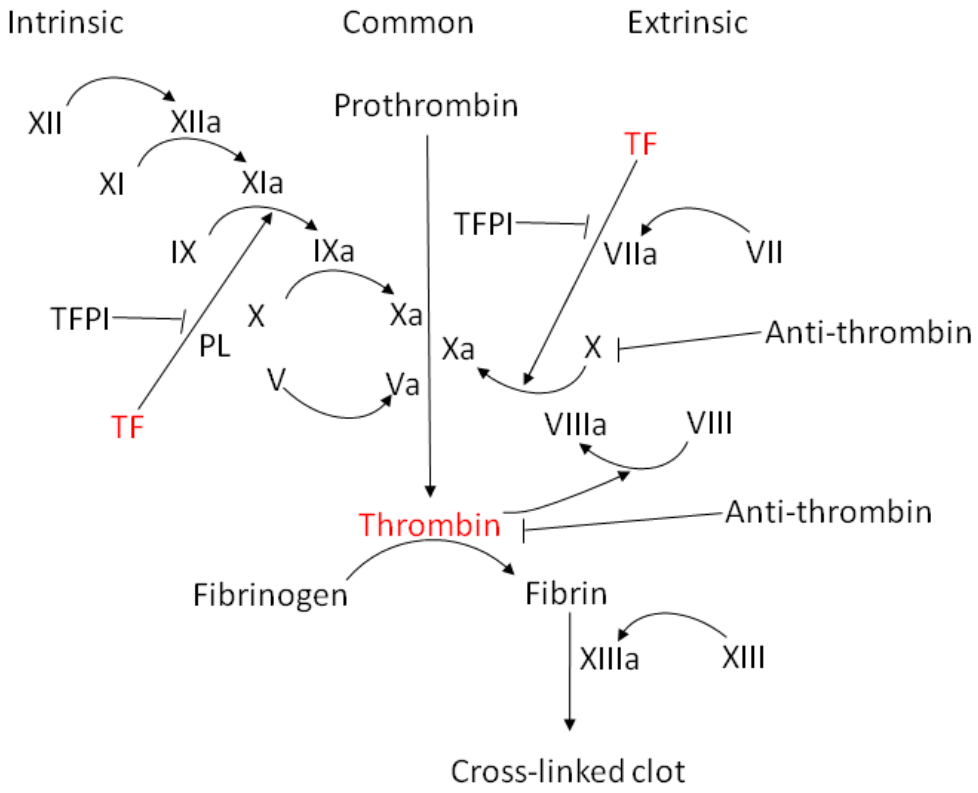

Figure 1.1: The coagulation cascade

Vessel wall damage initiates the intrinsic coagulation cascade where each activated protease is responsible for activating the next. The extrinsic pathway supports and augments the activity of the intrinsic pathway and leads to increased thrombin activation. The co-factor TF is essential to the cascade as it is required for activation of thrombin, the key protease responsible for thrombus formation.

thrombospondin (TSP), as well as bridging between proteins and platelets, and between platelets and the vessel wall lead to an increasingly stable clot that is resistant to dissolution[6].

1.1.5 Fibrinolysis

During coagulation, release of tissue type-plasminogen activator (tPA) from the endothelium converts plasminogen to plasmin and facilitates the initiation of clot breakdown[6]. The activity of plasmin is mitigated by its interaction with α-antiplasmin which is directly crosslinked within the fibrin network and therefore influences the degree and location of fibrinolysis. The tightly regulated nature of hemostasis is due to the balance that exists between pro-coagulant and platelet aggregation cues, 'resting-state' inhibition of coagulation, as well as pro-fibrinolytic and anti-fibrinolytic reactions. Following the rapid and efficient response to injury the clot is gradually reduced to allow for wound healing and tissue repair[6].

1.1.6 Coagulation and Cancer

First recognized by Trusseau in 1865[10], it has long been recognized that tumors instigate changes in the hemostatic system that function to support tumor progression[4, 11-14]. Tumor growth is associated with a global hypercoagulable state, platelet abnormalities and thromboembolism, often leading to patient mortality (reviewed in [15]). A definitive role for components of coagulation in primary tumor growth has not been identified, as tumor growth is equivalent in fibrinogen-deficient and wild-type animals[16, 17]. During metastasis however, coagulation factors play an important role in sustained cell arrest, tumor cell survival and extravasation[16-23].

1.2 The role of host systems during metastasis

1.2.1 Tumor cell invasion and intravasation

Excessive proliferation of neoplastic cells in a developing cancer leads to hypoxia and necrosis in the tumor microenvironment. Tumor and stromal cells react by secreting growth factors and cytokines such as colony stimulating factor (CSF)-1 and tumor growth factor-β (TGF-β), which are chemoattractants for immune cells[24]. Further host reaction

to the developing neoplasm leads to recruitment of mesenchymal stem cells, activated fibroblasts, endothelial precursors, dendritic cells, macrophages, monocytes, lymphocytes, leukocytes and mast cells[25, 26]. Initially, it is likely that this recruitment is a host defense mechanism, but the tumor is able to capitalize on the pro-growth factors and counteract the growth-inhibitory capabilities of the recruited cells[26]. It would be expected that an abundance of immune cells would be beneficial for the host, yet it often correlates with poor clinical prognosis[27, 28].

A major effect of the inflammatory response to tumor development is an increase in tumor invasiveness. Breast cancer cells cultured in macrophage-conditioned media, or co-cultured with macrophages, show a significant increase in invasive behavior in vitro[29, 30]. The resulting increase in matrix metalloprotease (MMP) activity was found to aid tumor cell invasion[30]. These results indicate that tumor cells can capitalize on the host immune response leading to increased invasiveness and subsequent metastasis.

Tumor-associated macrophages (TAMs) are often the most common immune cell in the tumor microenvironment and play an essential role in tumor metastasis. Using an in vivo model of mammary carcinoma, it was found that TAMs are most likely to be found at the margin of a primary tumor, with decreasing numbers upon imaging deeper into the tumor[31]. The few TAMs that were found in the tumor core were associated with blood vessels and were essential for tumor cell intravasation (Figure 1.2 a). Analysis of murine and clinical samples found that TAMs may guide breast cancer cells toward blood vessels through epidermal growth factor (EGF)-CSF-1 signaling, as cancer cells were often found in contact with perivascular macrophages. The density of these interactions in clinical samples correlated with the histological grade of the tumor and positively associated with the risk of distant metastasis formation[32]. It has also been noted that macrophages are often present at the site of basement membrane breach and tumor cell dissemination[33].

Neutrophils, lymphocytes and TAMs all express and secrete MMPs, which collectively can degrade every ECM protein. The association of these immune cells with the invasive border of a tumor leads to a degradation of the physical barrier that prevents tumor cell

dissemination. This degradation releases and activates many growth factors (TGFβ, tumor necrosis factor α (TNFα), Fas Ligand, heparin bound-epidermal growth factor and others) that are normally sequestered in the ECM[34, 35]. It is understood that a tumor is not a uniformly organized mass – each tumor cell will have differential access to nutrients, oxygen and tumor stromal components depending on its individual location[36]. Direct imaging of murine mammary tumor growth using a mammary window was able to visualize individual cells longitudinally and evaluate differences in their behavior depending on their initial location. It was found that those cells in close proximity to blood vessels showed increased migration and invasion and were more likely to spread from the primary tumor to the lung than those cells that did not have immediate access to the vasculature[36].

Immune cells are a key component of tumor stroma, but the most abundant stromal cell is the carcinoma associated fibroblast (CAF)[37] (Figure 1.2 a), which is also associated with an increase in tumor cell invasion. These fibroblasts have been recruited as normal fibroblasts and are activated to become myofibroblasts, or have been recruited as bone marrow derived cells (BMDCs) and differentiate into fibroblasts at the tumor site[38]. Using a 3-dimensional (3D) in vitro model of the epidermal/dermal microenvironment, it was found that invasion of squamous cell carcinoma (SCC) cells always followed a leading CAF[39]. This leading fibroblast was able to create a track in the Matrigel matrix through both protease- and force-mediated remodeling that the SCC cells would follow. The track was found to be necessary and sufficient for SCC cell invasion as removal of the fibroblasts after track formation still allowed SCC cells to invade. These SCC cells have not undergone an epithelial-mesenchymal transition (EMT) and are non-invasive. It had been questioned how tumors that maintained an epithelial phenotype were able to intravasate; this work illustrates that those tumor cells that are not inherently invasive are able to co-opt host cells in order to metastasize[39]. Components of the host coagulation system are also involved in regulating tumor cell invasiveness. TF is consistently upregulated in many human malignancies and is found to contribute to many facets of tumor aggressiveness[40, 41]. TF is expressed by tumor cells, often at high levels, but also by many host cells such as endothelial cells, TAMs and CAFs. The main function of TF

is to activate thrombin which potentiates clot formation, but thrombin is also essential for activating protease activated receptor (PAR)-1 and -2. Activation of PAR-1 expressed by tumor cells leads to increased tumor invasion and metastasis through induction of proteases and cell adhesion molecules[42].

1.2.2 Survival and arrest in the vasculature

The host coagulation system is known to play a significant role in tumor cell arrest and survival in the vasculature. Tumor cells activate or produce many components of the coagulation cascade such as thrombin, PAR-1, TF, fibrinogen, VWF, and platelet-activating factor (PAF), leading to a 'platelet mimicry' phenotype[43]. The hypoxic tumor environment increases TF expression by endothelial cells, TAMs and CAFs leading to thrombin production within the primary tumor. This 'pre-treatment' with thrombin increases tumor cell adhesion to platelets and the vascular endothelium following tumor cell intravasation[44].

Through expression of TF, tumor cells are able to exploit the host coagulation system to increase metastatic efficiency. Within five minutes of metastatic cell arrest in the lung there is evidence of tumor cell association with platelets and fibrin.[23] In an elegant series of papers, Palumbo et al.[16-19, 45] evaluated the interplay between metastatic cells and the individual components of coagulation. They found that loss of host fibrinogen significantly decreased lung metastasis formation, yet had no impact on the number of cells that originally arrested in the lung following experimental metastasis cell injection. Fibrinogen was essential for sustained adherence of tumor cells in the lung vasculature[17]. The role for fibrinogen in cancer progression appears restricted to metastasis however, as fibrinogen knock-out animals had reduced lung metastasis despite equivalent primary tumor formation in fibrinogen-null and wild type animals[16]. Evaluation of metastasis in animals with activation-resistant platelets (platelets present in normal number, but not able to be activated by thrombin, adenosine diphosphate, or other coagulation stimuli) showed a significant decrease in experimental and spontaneous metastasis, again due to reduced survival or retention in the lung vasculature[18]. Depletion of circulating natural killer (NK) immune cells prior to metastatic cell introduction resulted in equivalent

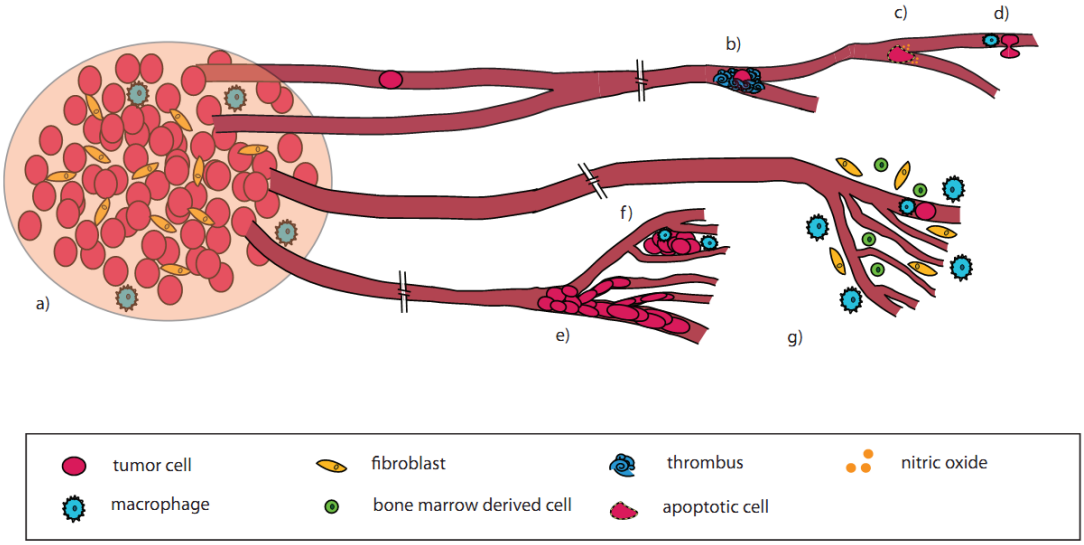

Figure 1.2: Host-tumor cell interactions during metastasis.

Interaction between metastatic tumor cells and the host environment in early stages of metastasis. (a) A primary tumor is infiltrated with host-derived macrophages and fibroblasts that aid in tumor cell invasion and intravasation. Upon arrest in a secondary site, tumor cells often stimulate formation of a thrombus (b), which provides adhesion contacts and protection from the host immune system. These arrested cells may undergo apoptosis due to release of nitric oxide from the vascular endothelium (c) or may extravasate, often with assistance from a host macrophage (d). Not all metastatic cells extravasate prior to initiating growth in a secondary organ, and intravascular micrometastases are found (e), especially in the lung. Extravascular micrometastatic growths (f) are also common, and often found to be associated with host macrophages. The site of metastatic growth is dependent on many factors, but formation of a pre-metastatic niche (g) is thought to direct and aid initial growth and survival of metastatic cells. (Reprinted from Kirstein JM and Chambers AF. Interactions of Normal Tissues and Systems with Metastatic Cells: Impact on Location, Survival and Growth. In: Experimental and Clinical Metastasis: A Comprehensive Review - In Press (Springer))

metastasis number in platelet mutant, fibrinogen knock-out and wild type animals, indicating that platelet- and fibrinogen-mediated thrombus formation protects tumor cells from NK cell surveillance in the lung vasculature[18]. The role of NK-mediated cell killing was strengthened through work on Factor XIII, which stabilizes fibrin and other ECM components through catalysis of crosslinkages[6]. Factor XIII was found to be essential in preventing NK cell immunosurveillance of tumor cells[45]. Crosslinking between tumor cells and platelets also contributes to firm arrest in the vasculature, as normal platelet bridging to the vasculature (in response to vasculature damage) assists to tether tumor cells within the vessel[14]. Thus, thrombi provide a physical barrier between tumor cells and circulating immune cells, and may actually lead to suppression of the immune response as TGFβ from platelets has been found to decrease the immunostimulatory factor interferon γ[46].

The formation of a thrombus at the surface of an arrested tumor cell has also been linked to increased metastasis through maintenance of cell adherence in the pulmonary vasculature (Figure 1.2 b)[4, 23, 47]. Metastatic cells protected in a fibrin clot were able to change from a rounded morphology and spread along the inside of a vessel. Those cells that showed stable adherence to the lung vasculature were able to form significantly more lung metastases than those prevented from spreading through treatment with anticoagulant agents[23]. In accordance with this, prevention of thrombus formation with heparin[48, 49] or hiruden[50] is linked with reduced pulmonary metastasis due to decreased cell retention in the lung.

Stable adherence of tumor cells to the vasculature upon arrest appears to be a major determinant of metastatic efficiency. Comparison of metastatic and non-metastatic cells injected into the circulation showed no difference in the original number of cells that arrested in the lung, however only those cell lines that had a metastatic phenotype were able to resist apoptosis and form micrometastases in the lung[51]. Tumor cell arrest is also influenced by host expression of P-selectin. Platelets isolated from P-selectin knock-out mice were unable to bind to tumor cells in vitro, and experimental metastasis assays found that there was a decrease in the initial seeding of the lung tissue in P-selectin-null animals[47]. Additionally, P-selectin was found to facilitate tumor cell tethering and rolling

along the pulmonary vasculature, but further binding by $\alpha_{IIb}\beta_3$ was required to stabilize tumor cell adhesion[52]. Integrin $\alpha_3\beta_1$ is also involved in tumor cell adhesion to the vascular endothelium through sections of exposed basement membrane. Adhesion and migration of tumor cells was also stimulated by binding of TF on tumor cells to tissue factor pathway inhibitor -1 (TFPI-1) on tumor associated vessels[53]. Tumor cell-associated thrombus formation may also increase metastatic cell survival in the vasculature, as activation of PAR-1 by thrombin leads to transmission of survival signals and prevention of apoptosis[54]. Additionally, many growth and survival factors are released from platelets upon activation and are therefore present within thrombi[55]. Tumor cells are able to bind to the provisional matrix provided by a fibrin clot thereby increasing metastasis (Figure 1.2 b)[16, 56, 57]. Further, plasmin-mediated clot dissolution may aid tumor cells with the next step in metastasis – extravasation from the host vasculature.

1.2.3 Extravasation and growth initiation in secondary tissue

Compared with the other steps in metastasis, relatively little is known about tumor cell extravasation at a secondary site. Using cell accounting techniques Luzzi, et al.[58] found that the majority of B16F1 murine melanoma cells had extravasated from the liver vasculature within 3 days of cell injection[58]. Importantly, very few of these cells went on to form micrometastases (2%) and even fewer were able to form macrometastases (0.02%). Two weeks following tumor cell injection, over one-third of injected cells remained in the liver as solitary, extravasated cells and 95% of those identifiable cells were not apoptotic or proliferating (as determined by histological staining for TUNEL and Ki67). The low rate of metastasis yet high level of extravasation in this model indicates that in the liver, extravasation may not be an essential part of metastatic inefficiency. Additionally, using the chick chorioallantoic membrane (CAM), Koop, S et al.[59] found that nearly all B16F1 cells were able to survive and extravasate following arrest. Tissue inhibitor of metalloproteinases-1-overexpressing B16F1 cells were poorly metastatic, and yet they were still able to successfully extravasate in the chick CAM model[59]. Using *ras*-transformed and control fibroblasts, it was also found that extravasation was independent of metastatic ability[60]. Nearly all *ras*-transformed

fibroblasts and control fibroblasts (89 and 96%, respectively) had extravasated from the chick CAM within 24 hours of initial injection. Additionally, migration of both cell types within the mesenchymal layer was equivalent, despite having differential invasion capabilities in vitro[60].

Direct visualization of tumor cell extravasation was performed recently in a murine model of brain metastasis[61]. Using a cranial window, single cancer cells were visualized throughout arrest and extravasation. MDA-MD-435 cells were found to arrest in microvessel branch points and extravasate as single cells. These cells began to proliferate only after successful extravasation and only when extravasated cells maintained contact with an abluminal endothelial cell of a brain capillary[61].

Study of metastasis to the lung vasculature shows a distinct difference from that seen in the liver and chick CAM, however. Using the 4T1 murine mammary carcinoma cell line it was found that these cells arrest in the lung as individuals attached to the vascular endothelium. The cells were able to form small colonies within three weeks, some entirely maintained within the vasculature. The colonies were then able to extravasate as micro or macrometastases[62]. Further to this, fewer than 2% of HT1080 cells had extravasated from the lung vasculature within 24 hours of tumor cell injection, and were found to form colonies within the lung vasculature within three days. These colonies showed tumor cells that projected outwards from the central focus as 'strings' following within the capillaries (Figure 1.2 e)[63]. Analysis of experimental metastasis of B16F10 melanoma cells in the mouse lung found that the majority of injected cells had extravasated, with no identifiable clusters or single cells within the pulmonary vasculature within 4 days of injection[64]. Using an orthotopic prostate cancer model, however, the majority of metastatic tumor cells and tumor cell clusters were found within the vasculature of both the liver and the lung[65]. Taken together, these data indicate that the role of extravasation in successful metastasis formation may be specific to the model, cell type and secondary organ of study.

It is known that arrest of tumor cells is associated with the formation of a fibrin clot at the arrested cell site. These clots do not persist indefinitely – clot dissolution is mediated by

the powerful protease plasmin[56]. This clot breakdown may aid tumor cell extravasation through activation of MMPs and other proteases. Additionally, activated platelets are able to increase vascular permeability; platelet secretions and clot dissolution can both result in retraction of the endothelium to assist immune cell colonization at inflammatory sites, but during metastasis may enable tumor cell extravasation[21]. Tumor cells that express high amounts of urokinase type plasminogen activator (uPA) tend to be more aggressive and metastatic (reviewed in [66]). Clinically, high levels of uPA, uPA receptor (uPAR), plasminogen activator inhibitor (PAI)-1 and PAI-2 are linked to poor prognosis and increased metastasis development[67, 68].

1.2.3.1 Pre-metastatic niche formation

The site of metastatic cell arrest and growth has been debated for some time – from Stephen Paget's theory of 'seed and soil' where the tumor cell (seed) must arrest in a permissible secondary tissue (soil) in order to develop into a tumor[2, 69]. This century-old theory still has merit as metastatic cells grow in different tissues depending on the tumor type they originated from. A type of hospitable 'soil' has been identified as a pre-metastatic niche. These regions of secondary tissue show recruitment of clusters of BMDCs and hematapoietic progenitor cells (HPCs) colonizing a distant organ prior to the arrival of tumor cells. The primary tumor stimulates pre-metastatic niche formation through secretion of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), which recruit VEGF receptor 1 (VEGFR1)-positive cells. PlGF in particular increases the proliferation of fibroblast-like cells and stimulates their production of FN[70]. BMDCs expressing VEGFR1 and $\alpha_4\beta_1$ integrin arrest in regions of increased FN synthesis and secrete MMP-9 which may degrade the basement membrane to allow extravasation of more BMDCs and/or metastatic cells. They are also found to express Id3, which is involved in proliferation and mobilization of HPCs from the bone marrow and maintains an activated state within the BMDC clusters. These clusters alter the local microenvironment and activate integrins and chemokines such as stromal derived factor-1 (SDF-1). This activation leads to further recruitment of BMDCs and increased attachment, survival, and growth of tumor cells (Figure 1.2 g)[71]. Pre-metastatic niche formation can also be directed by platelet aggregation[72]. At a site of endothelium

disruption, platelet activation was essential for recruitment of BMDCs, which adhere to P-selectin and $\alpha_{IIb}\beta_3$ on the platelet surface, rather than to exposed ECM[73]. Additionally, SDF-1 released from platelets leads to ongoing retention of BMDC and tumor cell arrest[73].

Interestingly, the location of pre-metastatic niche formation was found to be driven by factors released from the primary tumor, with different tumor types stimulating niche formation in differing locations. Injection of conditioned media from one tumor type was able to confer its metastatic pattern onto another tumor type[71]. The specific factors involved in this stimulation have not been fully elucidated, but intriguing work with human breast cancer cells has identified osteopontin as a major player[74].

1.2.3.2 Osteopontin

Osteopontin (OPN) is a secreted, integrin-binding glycophosphoprotein that is involved in many cellular functions including adhesion, invasion, migration, and prevention of apoptosis (reviewed in [75, 76]). Analysis of patient plasma has found that high OPN levels correlate with poor prognosis in breast,[77] prostate,[78] lung[79, 80] and ovarian[81] carcinomas. Additionally, overexpression of OPN has been detected in melanoma, stomach and colorectal cancers (reviewed in [82]). OPN expression by tumor cells increases malignant behaviour and extensive work has been done on the role of OPN and metastasis, particularly in breast cancer models (reviewed in [76]). OPN has been found to increase spontaneous pulmonary and lymphatic metastasis[83] and the effect of OPN has been linked to β_3 integrin-mediated signalling[84]. OPN has also been linked to increased tumor angiogenesis and plays an important role in immune and inflammatory responses[85].

Investigation of the role of the tumor microenvironment on tumor progression identified OPN as a protein involved in BMDC mobilization thereby increasing tumor development. McAllister, SS et al.[74] investigated the effect of an actively growing primary tumor on the growth of an otherwise indolent tumor on the contralateral side. It was found that OPN expression from the growing tumor stimulated BMDC mobilization and colonization of both tumor sites, leading to increased growth and progression[74]. This effect of a primary tumor indicates that the full interaction between a tumor and host

is exceptionally complex and involves the interaction between primary tumor, host systems, and metastatic cells.

1.2.4 Angiogenesis and sustained growth

Sustained primary tumor and metastatic growth beyond ~1mm^3 requires the recruitment of a blood supply[86]. Vascularization of tumors promotes growth by providing oxygen and nutrients and increases metastasis by providing an entry point into the circulation. Normal tissues undergo angiogenesis during development, wound healing and tissue regeneration, through a tightly regulated system leading to structured, hierarchical branching of vessels[87]. This regulation is due to coordinated expression levels of pro- and anti-angiogenic factors, and is lost during tumor neo-vascularization. Deregulated angiogenesis in a tumor is due to an imbalance between pro- and anti-angiogenic factors in the tumor microenvironment. The over-expression of pro-angiogenic factors VEGF-A, angiopoietin (Ang)-2, basic fibroblast growth factor and TGFβ leads to constant stimulation of angiogenesis and a reduction in stabilized vessels. This leads to poor tissue perfusion, high vasculature permeability and chronic inflammation and an increase in metastasis due to ease of metastatic cell entry into the vasculature[88]. Thus, the tumor vasculature is characterized by highly tortuous dysfunctional vessels due to improper regulation of angiogenesis[89].

Expression of VEGF-A by tumor cells, macrophages[90-92], neutrophils[93], platelets[94], fibroblasts[95] and endothelial cells[96] tips the balance of pro- and anti-angiogenic factors in the tumor microenvironment and leads to widespread activation of angiogenesis. VEGF-A is elevated in response to hypoxia and inflammation, which are common in during tumor formation. Solid tumors tend to have a hypoxic core due to poorly functioning vasculature leading to constant stimulation of pro-angiogenic factors such as VEGF-A[97].

The process of angiogenesis in the metastatic setting is thought to proceed through similar mechanisms as seen in the primary tumor. Initial growth of a micrometastasis is halted without the recruitment of a blood supply, leading to a functionally dormant metastasis with balanced levels of proliferation and apoptosis[98]. Upon activation of the 'angiogenic switch'[99] tumor cells and macrophages present at the metastatic site stimulate

expression of VEGF-A leading to the same cascade of angiogenic events as seen in the primary tumor setting[98].

Blood clot formation at the metastatic site provides further angiogenic and growth signals as platelet activation results in the release of many growth and pro-angiogenic factors such as VEGF, platelet derived growth factor (PDGF), Ang-1, TGFβ, insulin-like growth factor 1, EGF, and platelet-derived epidermal growth factor (PD-EGF). Additionally, thrombin activity is linked to increased angiogenesis through up-regulation of cathepsin-D which increases endothelial cell growth, migration and tube formation in vitro[100]. Thrombin may also play an important role in angiogenesis through induction of VEGF-A in tumor cells[101] and platelets[102], as well as Ang-1 and -2 from platelets[103] and endothelial cells[101] respectively.

1.2.5 Host-tumor interactions as therapeutic targets

As described above, there is extensive interaction between the primary tumor, the host and developing metastases. There is the potential for the tumor to exploit host systems and augment tumor progression; yet therapeutic interventions to prevent this exploitation have not been well investigated. Full understanding of the complex relationship that exists between tumor, host, and metastases is required to allow development of new types of host-defence therapeutics. An example of treating the host to treat the tumor lies in clinical modulation of hemostatic targets. Given the robust interaction between metastatic cells and the host hemostatic system[16-23] it is not surprising that treatment of patients with anti-coagulants has a role in metastatic progression. Of great clinical interest is the role of P-Selectin inhibition on tumor progression as murine models of metastasis have shown that treatment with several types of low molecular weight heparin (LMWH) leads to inhibition of metastasis through binding to P-Selectin and preventing platelet adhesion to tumor cells (reviewed in [55]). Several other pharmacologic means of inhibiting tumor cell interaction with coagulation factors include antibody-mediated inhibition of integrin function, specific inhibition of thrombin, targeting of PARs, and inhibiting platelet aggregation, though the anticancer efficacy of antiplatelet agents has not been tested in clinical trials (reviewed in [104]).

1.3 Pharmacologic modulation of hemostasis

Modulation of hemostasis through pharmacologic intervention enables treatment of many clinical concerns, such as deep vein thromboses and pulmonary embolism[105]. Treatment of patients with venous thromboembolism (VTE) with anti-coagulants can significantly reduce the risk of stroke and myocardial infarction and their associated morbidity and mortality. All anti-coagulants carry the risk of excessive bleeding and treatment must be monitored closely[105]. Alternatively, major surgical intervention (cardiopulmonary bypass, liver resection, hip replacement, others) has the concern of excessive blood loss during surgery[106]. Treatment of surgical patients with anti-fibrinolytic agents has been shown to significantly reduce the number of blood transfusions needed during surgery as well as the need for re-operation to control bleeding[107]. The mechanism of action of anti-coagulant and anti-fibrinolytic agents, their clinical use, and their role in cancer is discussed below.

1.3.1 Heparin and anti-coagulant therapies

Heparin molecules are long, unbranched polymers of glucosamine and galacturonic acid and are highly heterogeneous in length[105]. The molecular weight of unfractionated heparin (UFH) ranges from 3000 to 30,000 daltons, but only a third of heparin molecules have the high-affinity pentasaccharide required for anti-coagulant activity[105]. Heparin drastically inhibits thrombin activity by binding to antithrombin (AT) and increasing its affinity for thrombin and factor Xa. Long-chain heparin molecules can also directly bind and inhibit thrombin activity. Low molecular weight heparin (LMWH) is derived from heparin through enzymatic or chemical depolymerisation and has a smaller and more narrow size range (1000 – 10,000 Da)[105]. The major difference between UFH and LMWH is reduced interaction between LMWH and thrombin, but LMWH maintains its anti-coagulant activity through interaction with AT. LMWH has several advantages over UFH, including an extended half-life and more predictable anti-thrombotic dose response[108]. Recent clinical studies of UFH and LMWH treatment of VTE in cancer patients has identified a significant increase in survival following extended treatment[108], especially with LMWH. Importantly, this effect may be in-part due to mechanisms outside of anti-coagulation[108, 109].

1.3.2 Anti-coagulants and cancer

Cancer patients are prone to development of VTE, leading to significant morbidity and mortality[15, 110-112]. The hypercoagulable state in cancer patients is due to a number of factors including i) alterations in blood flow due to patient immobility, tumor-induced mechanical blockage, or thrombocytosis; ii) impaired vessel integrity due to extensive angiogenesis, tumor cell-induced vascular damage (during invasion and metastasis) or poorly functional, leaky tumor vasculature; iii) procoagulant activity of tumor cells[14, 15, 41, 110-114]. Treatment of cancer patients with anticoagulants for relief of VTE has led to the important discovery that LMWH therapy can improve patient survival, especially in those patients who did not have identified metastatic disease at the time of study enrolment[115]. Interestingly, this survival benefit may not be due to prevention of coagulation, as treatment with the non-heparinoid anti-coagulant coumarin did not lead to a similar survival benefit[116, 117].

Indeed, inhibition of coagulation is able to decrease metastasis as treatment with the thrombin-specific inhibitor hiruden leads to a significant inhibition of experimental lung metastasis[50]. Heparin has been definitively shown to alter early events in metastasis, specifically the sustained arrest and survival of tumor cells in a secondary capillary bed[23, 49]. Inhibition of fibrin and platelet deposition around tumor cells leaves them vulnerable to NK-cell mediated killing[18, 19], but the multi-faceted role of heparin in vivo indicates that non-anticoagulant mechanisms may also play a role in heparin-mediated reduction of metastasis. For example, heparin can block P- and L-Selectin, which has been shown to reduce sustained metastatic cell arrest[117]. Heparin also affects the activity of growth factors, causes the release of TFPI, inhibits angiogenesis, alters integrin interactions, and modulates protease activity[112, 118]. Thus, the effect of heparin on tumor progression is multi-faceted and has led to specific pre-clinical investigation of the effect of LMWHs on metastasis to isolate the effect of individual heparin preparations. The LMWHs tinzaparin, dalteparin, nadroparin and, enoxaparin have potent anti-metastatic effects when administered prior to tumor cell delivery (reviewed in [119]). Also, derivation of a LMWH with no anticoagulant ability was still able to protect against experimental metastasis of B16F10 melanoma cells[109]. Together, these data indicate the inhibition of

coagulation can lead to significant inhibition of metastasis, but the effect of the widely-used anti-coagulant heparin may have a multitude of mechanisms as compared to thrombin-specific inhibitors.

1.3.3 Aprotinin and anti-fibrinolytic therapies

Aprotinin is a broad-spectrum serine protease inhibitor used clinically for several decades to reduce intra-operative blood loss[120]. It binds to and inhibits trypsin-like enzymes such as trypsin, chymotrypsin, plasmin, kallikrein, elastase, and plasmin activator, with decreasing affinity[121]. It is the inhibition of plasmin-mediated dissolution of thrombi that is thought to lead to reduced blood loss[122], but aprotinin also demonstrates anti-inflammatory and platelet preserving effects[123] making it an ideal therapeutic during invasive surgery. Recently, however adverse clinical reactions following aprotinin treatment have been identified. A clinical study of 781 patients who received aprotinin as compared to the lysine analogues tranexamic acid (TXA) and ε-amino caprioic acid (EACA) found to a slight increase in mortality (6.0% in aprotinin treated vs. 3.9 and 4.0% in TXA and EACA treated respectively) within 30 days of surgery [124]. This finding is controversial as robust meta-analysis of 52 clinical studies (over 7000 patients) found no such increase[107]. Despite this disparity, aprotinin has been removed from the clinic and other anti-fibrinolytic agents such as EACA and TXA have received more widespread use. The lysine analogue EACA and TXA are more selective protease inhibitors and are not as effective at reducing surgical blood loss during cardiac surgery as aprotinin[107, 125]. Additionally, investigation of inducible nitric oxide synthase (NOS) expression from bronchial epithelial cells found that EACA[126] and TXA[127] was not as effective as aprotinin at reducing nitric oxide (NO) release, indicating that aprotinin exhibits other clinically-relevant organ protecting effects that may not be present following treatment with other anti-fibrinolytic agents[123, 128-131].

1.3.4 Anti-fibrinolytics and cancer

Routine treatment of patients undergoing invasive surgery with anti-fibrinolytics has led to investigation of the effect of these agents following cancer-related surgery[132]. Similar to the observed effect following cardiac surgery, aprotinin treatment reduced peri-operative blood loss in meningioma, femoral osteosarcoma and bladder carcinoma

surgery[133] as well as following liver resection for treatment of colorectal metastases[134]. Aside from the significant effect on blood loss during surgery, the role of protease inhibition by anti-fibrionlytic agents on the tumor cell biology has also been investigated.

Two distinct hypotheses exist for the potential effect of protease inhibition on tumors and metastatic spread. The first indicates that due to the extensive role of proteases in angiogeneisis and growth factor activation as well as tumor cell invasion and extravasation, inhibition of proteases should decrease metastasis by preventing these essential processes. Alternatively, inhibition of plasmin-mediated clot dissolution could promote cancer cell survival and metastasis by stabilizing the interaction between intravascular tumor cells and thrombi[23, 135], as illustrated in Figure 1.3. Indeed pre-clinical data can be found to support both hypotheses. The role of aprotinin on clinical cancer progression has also been investigated. Treatment of patients undergoing liver resection for treatment of colorectal metastasis showed a significant increase in patient survival one year following surgery, but this improvement was not evident 5 years following surgery as survival rates were then equivalent in aprotinin- and placebo-treated patients[132].

1.3.5 Summary: modulation of hemostasis in cancer patients

Therapeutic modulation of hemostasis in cancer patients is complex and must be closely monitored, especially given the volatile host background. Importantly, treatment of a hemostatic imbalance may have effects beyond coagulation and fibrinolysis and may lead to promotion or inhibition of tumor progression. Detailed understanding of the interplay between tumors and host systems will allow for more effective treatment options and ensure that treatment of one facet of disease does not aggravate or promote another.

Related to this approach to cancer research is investigation into the ability of a host to prevent or restrict tumor progression in order to identify potential targets that could augment this host response. Specifically, analysis of molecular interactions between tumor and host or understanding the phenomenon known as concomitant tumor resistance, where the presence of a primary tumor can restrict the growth of secondary tumors or metastases, could lead to a promising new generation of treatment options.

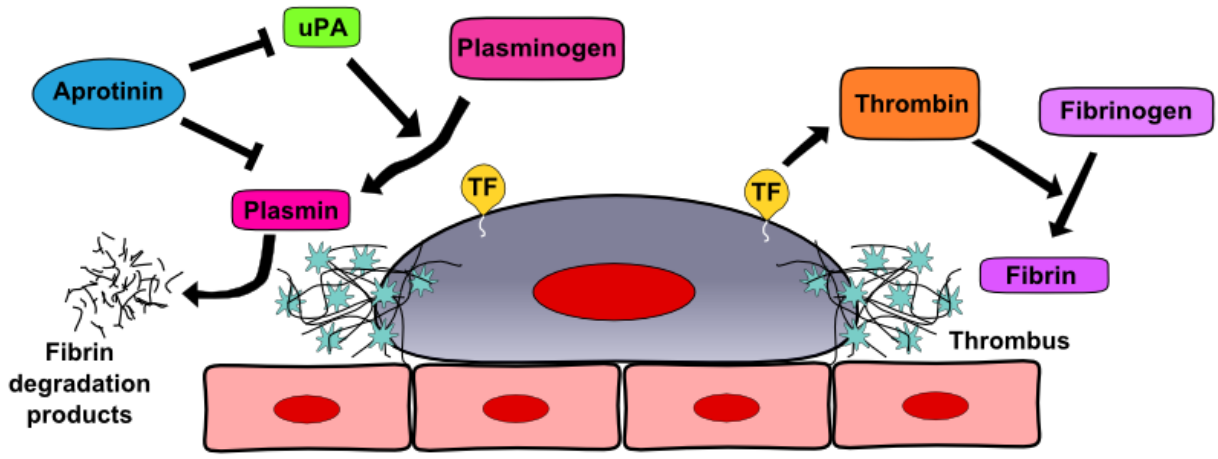

Figure 1.3 Interaction between an arrested tumor cell and cell-surface thrombus.

An arrested tumor cell may stimulate the formation of a thrombus through expression of TF on the cell surface. Clot formation is normally balanced with fibrinolysis by plasmin, however in the presence of the serine protease inhibitor aprotinin, fibrinolysis may be delayed, leading to prolonged interaction between tumor cells and thrombi.

1.4 Host-mediated inhibition of metastasis

Successful metastasis formation results when tumor cells are able to exploit and avoid natural host defenses. Yet metastasis is an exceptionally inefficient process[2], indicating that the host is capable of preventing progression of the majority of metastatic cells. The mechanisms behind this prevention are largely unknown, yet several interesting examples of host triumph over tumor have been established.

1.4.1 Molecular interactions limiting tumor metastasis

Following tumor cell arrest in the liver vasculature, NO is released and induces apoptosis in B16F1 cells[136]. B16F1 cell arrest in the pulmonary vasculature was also found to lead to an endogenous NOS-dependent release of NO. NO may represent a natural host defense mechanism as it triggers apoptosis in melanoma cells and reduced the growth of metastatic tumors (Figure 1.2 c)[137]. Accordingly, comparison of metastatic and non-metastatic melanoma cells following arrest in the murine lung showed that non-metastatic cells were unable to survive in the pulmonary vasculature. Within 8 hours of tumor cell injection, non-metastatic cells had apoptosed and were cleared from the lung, whereas metastatic cells persisted and were able to form metastatic colonies within 7 days[51].

Given the extensive interaction between tumors and the host, there is the potential to alter the microenvironment to create an anti-tumor rather than pro-tumor interface. It has been proposed that the large number of TAMs present in tumor stroma could be 're-educated' to target tumor cells[138]. Using nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, tumor cells are able to keep TAMs in an immunosuppressive state. By introducing a dominant negative inhibitor of nuclear factor κB kinase β into bone marrow derived macrophages, TAMs became tumoricidal through release of NO and through promotion of NK cell-mediated killing[138]. The extensive interaction between TAMs and metastatic cells throughout invasion and extravasation as discussed earlier (section 1.2.6) illustrates the great potential for manipulation of TAM activity to reduce tumor progression.

Normal tissue structure and function is maintained through proper ECM adhesion and tissue polarity. In breast and melanoma tumor development, dysregulation of cell adhesion represents an initiating step in tumor formation[139, 140]. Therefore the effect of re-establishing proper tissue architecture and adhesion in tumor tissues has been investigated[141]. It was found that restoration of proper integrin signaling within a 3D culture setting led to phenotypic reversion of breast cancer cells. Without alterations to tumor cell genotype, tumor cells were induced to form normal breast structures. Metastatic breast cancer cells could also be reverted to a non-malignant phenotype in 3D culture following treatment with anti-integrin antibodies[142]. The global switch in cellular behavior as a direct result of modulation of environmental interaction indicates the powerful role that the tumor stroma and microenvironment has on tumor development and progression and illustrates that many treatment options are available beyond direct targeting of tumor tissue.

1.4.2 Concomitant tumor resistance

It is a clinically recognized phenomenon that removal of a primary tumor from the patient can be followed by an explosive outgrowth of previously undetected metastases[143]. It appears that the presence of a primary tumor can hold secondary metastases `in check` by some unknown mechanism which is removed upon excision of the original tumor[143, 144]. Indeed, the ability of a primary tumor to restrict the growth of a second tumor implant was first recognized by Ehrlich in 1906[145] and was thought to occur by an immunological mechanism and was therefore termed concomitant tumor immunity[144]. The study of concomitant tumor immunity has led to many significant tangential discoveries that have improved the understanding of immunology[146], angiogenesis[147], and tumor growth[86, 148]. When first identified, three possible hypotheses were presented that could lead to CTR (reviewed in [144]).

1) Concomitant tumor immunity – the presence of a primary tumor induces an immunological response against a metastasis or secondary tumor.

2) Production of anti-mitotic or otherwise growth restricting compounds by the primary tumor that inhibit the development or progression of a second inoculum or metastasis.

3) Athrepsia – depletion of essential nutrient or factor by the tumor restricts the growth of a second tumor mass.

The immune system has been found to play an important role in inhibition or rejection of a second tumor, but only in highly immunogenic tumor types. The protection afforded by this immune reaction is tumor type specific – that is the host can only reject the same tumor cells to which it had been previously exposed, and only if the host had mounted an immune response upon first exposure[144].

Extensive work on concomitant tumor resistance by Judah Folkman's group has led to the current understanding of angiogenesis and the identification of angiogenesis inhibitors which have since been developed into therapeutic agents[86, 147]. The underlying hypothesis for his work was that tumor angiogenesis is based on a balance of pro- and anti-apoptotic regulators and that if these regulators exit the tumor and enter the serum of a tumor-bearing host there could be distant effects on other tissues, including secondary tumors[86]. Angiostatin, an internal cleavage fragment of plasminogen, was identified and it was found that it was able to suppress the growth of lewis lung carcinoma (LLC) metastases due to inhibition of angiogenesis at the metastatic site[147]. Importantly, tumors were found to make both pro- (VEGF) and anti-angiogenic factors (TSP, endostatin, angiostatin) with the local balance tipping toward increased angiogenesis and growth[147]. However, differences in stability of pro- and anti-angiogenic factors gives rise to an anti-angiogenic environment in regions distant to the primary tumor[86].

The third hypothesis, depletion of nutrients is responsible for metastatic inhibition, is supported by historic literature that found that the Gompertzian growth pattern of primary tumors occurred simultaneously in distant metastases, despite their significantly smaller size[149]. That is, the growth of a tumor slows as the tumor reaches a large size and as the primary tumor slows the secondary metastases also slow. In the absence of the primary tumor, these metastases would continue to grow, therefore it was thought that a systemic depletion of some essential factor led to the communal slowing of all tumors in the host[149]. Additionally, many cancer patients[150] and pre-clinical models[151] show extensive cachexia – the systemic wasting of the host with weight loss, fatigue, muscle atrophy,

weakness and a loss of appetite. It is possible that the tumor is usurping nutrients available to the host leading to the starvation of host tissues and developing metastases[151].

Following the identification of anti-angiogenic molecules and understanding of their ability to restrict the development of secondary tumors, further investigation into the non-immunogenic mechanisms of concomitant tumor resistance has slowed, despite many intriguing questions which remain unanswered.

1.5 Conclusions and Rationale

The study of tumor biology and metastasis has long been investigated from the perspective of the individual tumor cell. However, the importance of tumor cell interactions with host cells and systems has also been recognized. Tumor cells are unable to form metastases without interaction with many microenvironments – from the primary tumor stroma, through the host vasculature and host coagulation systems, to an entirely new environment in a secondary organ. The metastatic cell's ability to survive and proliferate in each of these new environments depends on its ability to influence and often exploit the host. Fundamental to this is the interaction of tumor cells with the host hemostatic system. Understanding the interplay between tumor progression and hemostasis and the potential for the host to circumvent or prevent metastasis is essential for understanding metastatic disease. This understanding will provide improved treatment options through augmentation of beneficial host responses to the tumor and will improve the chances for and successful patient treatment.

1.6 Objectives

The complex interplay between a tumor and a host is not fully understood, but it should be acknowledged that research focusing exclusively on the intrinsic properties of tumor cells is insufficient. Therefore, the overall objective of this work was to investigate the interactions between tumor cells and the host and the role that host systems play in supporting or inhibiting tumor development and progression. To this end, three specific objectives were undertaken.

1) To investigate the interaction between coagulation and metastatic cells and determine if global modulation of fibrinolysis would encourage or inhibit metastasis.

2) To investigate the effect of a murine melanoma primary tumor on secondary metastasis development and the role of the host coagulation system in this effect.

3) To identify the effect of a breast tumor on metastatic development and to resolve the conflicting ideas of concomitant tumor resistance and pre-metastatic niche formation in a breast cancer model.

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Chapter 2

2 Effect of anti-fibrinolytic therapy on experimental melanoma metastasis

2.1 Synopsis

Anti-fibrinolytic agents such as aprotinin and EACA are used clinically to decrease peri-operative bleeding. Use of these treatments during cancer-related surgeries has led to investigation of the effect of fibrinolysis inhibition on cancer cell spread. The ability of aprotinin to reduce proteolytic activity of proteases required for metastasis suggests that it could have an anti-metastatic effect in patients undergoing tumor resection. However, many metastatic cells in the vasculature of a secondary tissue are associated with a micro-thrombus. The association of tumor cells with thrombi has been shown to increase their survival; therefore inhibition of plasmin-mediated fibrinolysis might instead increase metastatic cell survival by enhancing the association between thrombi and tumor cells. The goal of this work was to determine the effect of anti-fibrinolytic treatment on experimental metastasis and to establish the role of coagulation factors in this effect. The metastatic ability of B16F10 melanoma cells was evaluated in vivo following cell or animal pre-treatment with aprotinin or EACA. Additionally, a novel in vivo technique was developed, to permit analysis of tumor cell association with thrombi in the lung microvasculature using confocal microscopy. Aprotinin and EACA treatment of mice resulted in a significant increase in lung metastasis. Aprotinin treatment increased the size of thrombi in association with cells arrested in lung capillaries. This study suggests that clinical use of anti-fibrinolytic agents for cancer-related surgeries could result in increased metastatic ability of those cells shed immediately prior to and during surgery, and that this approach thus requires further study.

2.2 Introduction

Anti-fibriniolytic agents are often used during invasive surgeries to minimize blood loss and transfusion requirements[1]. Of these agents, the serine protease inhibitor aprotinin had been most commonly used and is highly effective at reducing blood loss in patients undergoing cardiopulmonary bypass[2]. Aprotinin therapy leads to a significant reduction in requirement for blood transfusions[3], and reduces post-operative inflammation and platelet exhaustion, previously shown to improve patient survival[1, 4]. Aprotinin inhibits most serine proteases, having a weak effect on thrombin activity but a large effect on trypsin-like enzymes such as kallikrein, plasmin, plasminogen activator and trypsin[5]. It is the potent inhibition of plasmin that leads to reduction of surgical blood loss, through inhibition of plasmin-mediated fibrinolysis[4]. Recent clinical studies have indicated an increased risk of mortality and renal failure associated with aprotinin treatment during cardiac surgery[6, 7], leading to a cessation of its clinical use. Other anti-fibrinolytic agents, such as the lysine analogs EACA and TXA, specifically inhibit plasmin activity through binding to plasminogen and preventing its conversion to active plasmin enzyme[8]. EACA and TXA have also been used clinically and have been compared to aprotinin in clinical trials to investigate their effect on blood loss during cardiac surgery[1]. EACA and TXA have not been associated with an increase in mortality or other major side effects including myocardial infarction, pulmonary embolism, deep vein thrombosis, or renal failure, but also are not capable of reducing blood loss to the same extent as aprotinin at current doses used[1]. The use of anti-fibrinolytic agents during cancer surgeries has led to investigation of their role in cancer progression and spread[9, 10].

It is well-established that protease activity is required for tumor cells to form distant metastases[11]. Proteases are employed when metastatic cells invade through primary tumor stroma into the host vasculature, and again when tumor cells extravasate and infiltrate upon arrest in distant tissues[12]. Serine proteases, including plasmin, play a pivotal role in metastatic growth as they function to activate MMPs[13] and release growth factors[14], thereby propagating tissue remodeling and increased growth – both essential processes for metastasis. Additionally, serine proteases can stimulate integrin-mediated binding to ECM proteins and cell-signaling cascades by activation of PAR-2 and uPAR[14].

Therefore, anti-fibrinolytic therapy could theoretically provide substantial benefit to cancer patients, not only by reducing blood loss during surgery, but also by reducing the metastatic ability of tumor cells.

In contrast, it is also possible that metastasis could be promoted by the presence of coagulation factors and thrombi. Metastatic cells may be coated in activated platelets and fibrin when they arrest in the secondary site, and this coating has been suggested to aid in cell survival[15-17]. Additionally, metastatic cells have been shown to adhere to and migrate along the temporary fibrin matrix in thrombi[18, 19]. Many cancer types are known to secrete factors which activate platelets including TF, thrombin, and CP, suggesting that coagulation may provide a survival benefit to cancer cells[19, 20]. Activated platelets also secrete many growth factors, including VEGF, PDGF and platelet derived-endothelial cell growth factor[20-23], which can promote tumor growth and angiogenesis[19, 24, 25]. In vivo analysis of metastasis in animals with congenital coagulation disorders indicates that the host coagulation state significantly impacts metastasis, as animals with a genetic predisposition to thrombosis show increased lung metastasis, while animals with a FVIII deficiency akin to human hemophilia show reduced lung metastasis[26]. Hence, the major clinical benefit of anti-fibrinolytic therapy may also prolong the duration of cancer cell-thrombus interaction, thereby improving metastatic cell survival and increasing the number of metastases formed.

The use of anti-fibrinolytics in cancer-related surgeries[9, 10] has raised the question of possible the effects of plasmin inhibition on cancer progression and metastasis. It has been reported that anti-fibrinolytics may cause a significant increase[27, 28] or decrease[29-31] in metastasis formation in animal models. Importantly, clarification of this issue has been difficult due to lack of in vivo mechanistic information linking the action of the anti-fibrinolytic to the metastatic outcome.

Given the divergent literature on aprotinin and metastasis, coupled with the current clinical use of anti-fibrinolytics in cancer-related surgeries, thorough investigation of the effect of anti-fibrinolytic agents on metastatic cells is required. Here, we used a well-established model of experimental metastasis to determine the effect of aprotinin and

EACA treatment on metastasis, and we present the first report to directly examine the effect of anti-fibrinolytic agents on tumor cell-thrombus association.

2.3 Materials and Methods

2.3.1 Cell culture and transfections

B16F10 murine melanoma cells (American Type Culture Collection, Manassas, VA) were maintained in α-minimal essential media (Invitrogen, Burlington, Canada), supplemented with 10% fetal bovine serum (Sigma, Mississauga, Canada). The LacZ expression vector was generated by cloning the LacZ cDNA into the expression plasmid pcDNA3.1 (Invitrogen). Stable transfections were performed using Lipofectamine 2000 (Invitrogen) per the manufacturer's guidelines. For selection of stable transfectants, hygromycin (Invitrogen) was added to the medium at 1000 μg/mL. Colonies were allowed to develop and then were isolated and grown as clonal populations. Each population was screened for intensity and uniformity of X-gal staining, and ability to retain such characteristics in vitro without selective pressure. A single clone, B16F10-LacZ, which consistently stained with high intensity and uniformity, was chosen. B16F10-LacZ was passaged in vitro without selective pressure for 38 days, and maintained the original staining characteristics. Additionally, this clone was tested in vivo and was found to have similar ability to form primary tumors and metastases as the parental B16F10 cell line. Cells were routinely tested and confirmed to be free of mycoplasma contamination using the Mycoplasma Plus PCR primer set (Stratagene, Cedar Creek, TX).

2.3.2 In vitro growth assay

To determine the effect of aprotinin treatment on B16F10-LacZ cell growth under standard, anchorage-dependent, monolayer growth conditions, cells were plated at 5×10^4 cells/60 mm plate. Cells were maintained in regular growth media or growth media supplemented with 200 KIU/mL aprotinin (Trasylol ® Bayer Pharmaceuticals, West Haven, CT). Media on both control and aprotinin treated plates was refreshed every 48 hours. Every 24 hours for 8 days, triplicate cultures were trypsinized and viable cells counted using a hemacytometer. Doubling times were estimated during the exponential

growth phase, according to the equation: $D = t*\ln 2/\ln(C_t/C_i)$, where t is time (hours) C_t is cell number at time t, and C_i is cell number at initial time of plating.

2.3.3 Experimental metastasis model

For experimental metastasis assays, B16F10-LacZ cells were suspended in cold, sterile Hanks Balanced Salt Solution (HBSS, Invitrogen). Cells were injected in 100 μL via the lateral tail vein into female C57Bl/6 mice, as described [32], using 5×10^5 cells/mouse and 10-16 mice/group. All mice were 7 - 8 weeks of age at the time of injection and were cared for in accordance to the Canadian Council on Animal Care, under a protocol approved by the University of Western Ontario Council on Animal Care.

2.3.4 Quantification of lung metastases

To enable visualization of lung metastases, mice were euthanized at various time points following cell injection and whole lungs placed in phosphate buffer (0.1 M sodium phosphate monobasic, 0.1 M sodium phosphate dibasic, pH 7.3), on ice until all lungs were isolated. Lungs were stained with X-gal (Bioshop, Burlington, Canada) solution as described [33] to visualize LacZ-expressing cells. The total number of surface-visible metastases was determined on intact lung lobes using a stereomicroscope.

The number of B16F10-LacZ tumor cell foci present at early time points (1 and 4 hours following cell injection) was determined by separating individual lung lobes and imaging on an inverted microscope (Nikon, Canada), using tissue autofluorescence to visualize boundaries between foci. The number of tumor cell foci, defined as single cells or clusters of tumor cells with no intersecting normal lung tissue, was quantified in fifty random fields per lung by a blinded observer.

2.3.5 Drug treatment protocols

To determine the effect of anti-fibrinolytic treatment on metastasis, animals were pre-treated with 5000 KIU aprotinin, 60 mg EACA, or saline (vehicle) fifteen minutes prior to cell injection. To determine the effect of anti-coagulant therapy on metastasis, animals were pre-treated with 100 U UFH (Pharmaceutical Partners of Canada), fifteen minutes prior to cell injection. All treatments were intravenously injected in 100 μl volume.

For cell pre-treatment experiments, B16F10-LacZ cells were pre-treated in culture using a protocol adapted from Esumi, *et al.* [34]. Briefly, 70-80% confluent dishes were incubated with standard growth media or media containing 200 KIU/mL aprotinin at 37°C for 30 minutes. Cells were washed twice with control growth media at room temperature and prepared for injection.

2.3.6 Analysis of coagulation using confocal microscopy

To enable analysis of coagulation in vivo, the following confocal microscopy procedure was developed. B16F10-LacZ cells were labeled in vitro using 5-chloromethylfluorescein diacetate, Cell Tracker Green (CMFDA, Molecular Probes, Burlington, Ontario, Canada), according to the manufacturer's instructions. Ten minutes before cell injection, animals received intravenous injection of 0.12 mg AlexaFluor647-conjugated fibrinogen (Molecular Probes) in 80μl sodium bicarbonate, pH 8.3. At various time points following cell injection, animals were euthanized by an intraperitoneal injection of xylazine/ketamine (5.2 mg ketamine and 0.26 mg xylazine per 20 g body mass). The lung was cleared of blood by gravity perfusion of HBSS through the right ventricle of the heart. 'Flow through' perfusate left the body via a puncture made in the left atrium. Lungs were perfused for 2 minutes, excised and frozen at –80°C to allow for later analysis by confocal microscopy. To visualize, lungs were thawed in cold HBSS prior to transfer to 0.1% paraphenylenediamine in HBSS for 10 minutes. Lungs were assessed using a Zeiss LSM 410 confocal microscope, and Carl Zeiss LSM 3.99 software. The presence of tumor cells was determined using green fluorescence and the focal plane containing the largest volume of the tumor cell was identified. At that same focal plane, the lung then was scanned for red fluorescence to identify thrombi. The green and red fluorescence were quantified in pixels by a blinded observer using ImageJ [35], and the ratio of thrombus to tumor cell area was determined.

2.3.7 Histology

After X-gal staining and transfer to 10% neutral buffered formalin, lung samples were paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E). Tumor

cell-associated thrombi were quantified on 5 H&E stained sections through all five lobes of the lung from each animal. Counts were performed by a blinded, trained observer.

2.4 Results

2.4.1 Aprotinin treatment increased experimental lung metastasis

To determine the overall effect of aprotinin treatment on experimental lung metastasis, animals were treated with aprotinin or saline prior to intravenous injection of B16F10-LacZ cells. Fourteen days following cell injection, the total number of lung surface metastases was quantified. Aprotinin treated animals showed a significant increase in the number of B16F10-LacZ lung metastases (t-test, $p < 0.05$, Figure 2.1 a).

2.4.2 B16F10-LacZ cell growth is not directly affected by aprotinin

Given that the tumor cells would encounter aprotinin immediately upon administration into the lateral tail vein, it was unclear if the increase in experimental metastasis caused by aprotinin is cell autonomous, or if the effect is mediated by the host. Therefore, B16F10-LacZ cells were exposed to aprotinin in vitro and assessed for changes in growth characteristics. No change in in vitro growth patterns (lag phase, exponential growth initiation) was observed (data not shown), and no change in doubling time was found (Figure 2.1 b).

To determine if the effect of aprotinin was cell-autonomous, yet only evident after in vivo injection, cells were pre-treated with aprotinin or control media in vitro prior to their preparation for in vivo injection. No effect of pre-treatment with aprotinin on B16F10-LacZ cells was observed, as cells pre-treated in vitro with aprotinin or control media had equivalent numbers of lung surface metastases 14 days following cell injection (Figure 2.1 c).

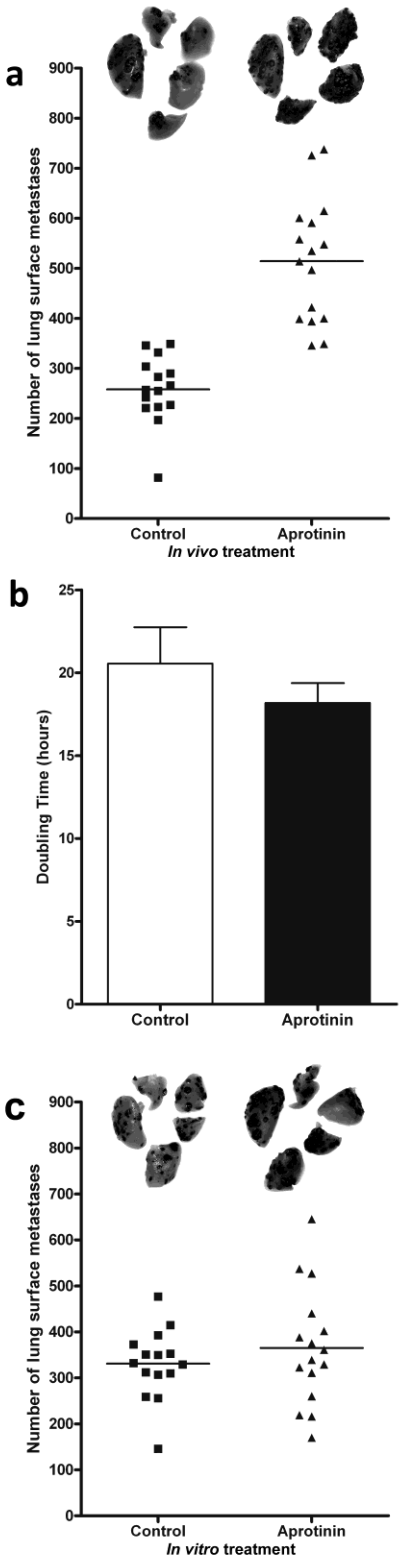

Figure 2.1 In vivo aprotinin treatment significantly increased the number of lung surface metastases.

a) Animals pretreated with saline or aprotinin received B16F10-LacZ cells via the lateral tail vein (n = 16/group) and the total number of lung metastases was quantified 14 days following cell injection (t-test, $p < 0.05$). Aprotinin treatment of B16F10-LacZ cells in vitro did not impact in vitro or in vivo growth. b) In vitro doubling time of B16F10-LacZ cells in standard growth medium supplemented with saline or aprotinin. Aprotinin treatment did not affect the growth rate in 2-D culture (t-test, $p > 0.05$). c) Number of lung surface metastases from mice injected with B16F10-LacZ cells that had been treated with saline aprotinin prior to their preparation for injection. Aprotinin treatment of cells alone did not impact the number of resultant lung surface metastases (t-test, $p > 0.05$). Columns, mean; bars, SE.

2.4.3 Aprotinin treatment increases persistence of cell foci at early time points

To clarify the mechanism by which aprotinin increased the number of B16F10-LacZ lung metastases, animals were treated with aprotinin or saline prior to tumor cell injection, and were sacrificed 1 or 4 hours following tumor cell injection. The mean number of surface-visible tumor cell foci per mm^2 was quantified following X-gal staining of whole lung tissue. It is known that fibrinogen is a key determinant of stable cell adhesion in the lung[18] and that the number of B16F10 tumor cells remaining in the lung decreases over early time points (1 hour through 24 hours)[36], therefore it was of interest to determine the effect of aprotinin on early cell survival or retention in the lung. One hour following tumor cell injection there was no difference in the number of LacZ-expressing tumor cells or cell foci in control or aprotinin treated animals, indicating that aprotinin did not affect initial arrest of B16F10-LacZ cells. However, four hours following tumor cell injection, significantly more B16F10-LacZ tumor cell foci remained in aprotinin-treated animals (t-test, $p < 0.001$, Figure 2.2 a and b). Examination of micrometastases revealed that aprotinin did not affect the growth of surviving tumor cells, as the size of individual micrometastases was unaltered by aprotinin treatment (Figure 2.2 c and d).

2.4.4 Histological analysis of thrombi associated with B16F10-LacZ cells

Tumor cell survival is known to be increased in the presence of a micro-thrombus[17], and given the potent inhibition of plasmin-mediated fibrinolysis by aprotinin, the effect of aprotinin on association of thrombi with B16F10-LacZ cells was investigated. To determine the effect of aprotinin on the presence of tumor cell-associated thrombi, animals were treated with saline or aprotinin and sacrificed 1 hour following cell injection. Quantification of histological sections (H&E) of X-gal stained lung tissue (Figure 2.3 a) showed a 2-fold increase in the number of thrombi found in association with tumor cells in aprotinin-treated animals (t-test, $p < 0.05$, Figure 2.3 b). To determine if the association between B16F10-LacZ cells and thrombi was essential for the establishment of lung metastases, animals were treated with the anti-coagulant, heparin

prior to melanoma cell injection. Heparin treatment significantly abrogated metastasis formation (Control: 365 ± 38.58, Heparin-treated: 1.571 ± 0.9221; n = 7, $p < 0.0001$).

2.4.5 Analysis of coagulation in lung capillaries using confocal microscopy

Given the effect of aprotinin on the persistence of tumor cell-associated thrombi in the lung 1 hour following cell injection, more detailed analysis of this association was warranted. Histological analysis was capable of identifying large thrombi in association with cells lodged in relatively large vessels; however, since the majority of lung metastases are formed from cells initially arresting in the microvasculature[36, 37], it is vital to examine this cell population. These cells are likely associated with micro-thrombi not readily visible using standard histological techniques and limited random sectioning. To address this need, we developed a novel technique which allows evaluation of coagulation around single or small clusters of tumor cells lodged in the lung microvasculature. Fluorescently labeled fibrinogen was injected prior to tumor cell injection and tumor cell-induced thrombus formation was quantified using confocal microscopy. With this procedure, we were able to clearly visualize thrombi in association with tumor cells at the earliest time point examined, 5 minutes (Figure 2.4 a), with the maximum thrombus size seen at 4 hours (Figure 2.4 b and c) and a decreased size at 6 hours (Figure 2.4 d). The presence of thrombi was not due to an accumulation of labeled fibrinogen from blockage of the capillaries with tumor cells, as injection of inert microspheres (20 μm, Polysciences, Pennsylvania), which also arrest by size restriction and cause vessel blockage [36, 38], did not initiate accumulation of fluorescence (Figure 2.4 f). Additionally, treatment of animals with heparin prior to tumor cell injection abrogated the presence of thrombi in association with tumor cells (Figure 2.4 g), indicating that the fluorescent fibrin(ogen) visualized is due to activation of the coagulation cascade and the presence of thrombi. To determine the effect of aprotinin treatment on thrombus size of individual capillary-arrested tumor cells, we used the same procedure to visualize fluorescent tumor cells in lungs of control and aprotinin treated mice using confocal microscopy. The presence and size of thrombi in micro-vessels was quantified for tumor cells visible on the surface of whole lung lobes isolated 4 hours

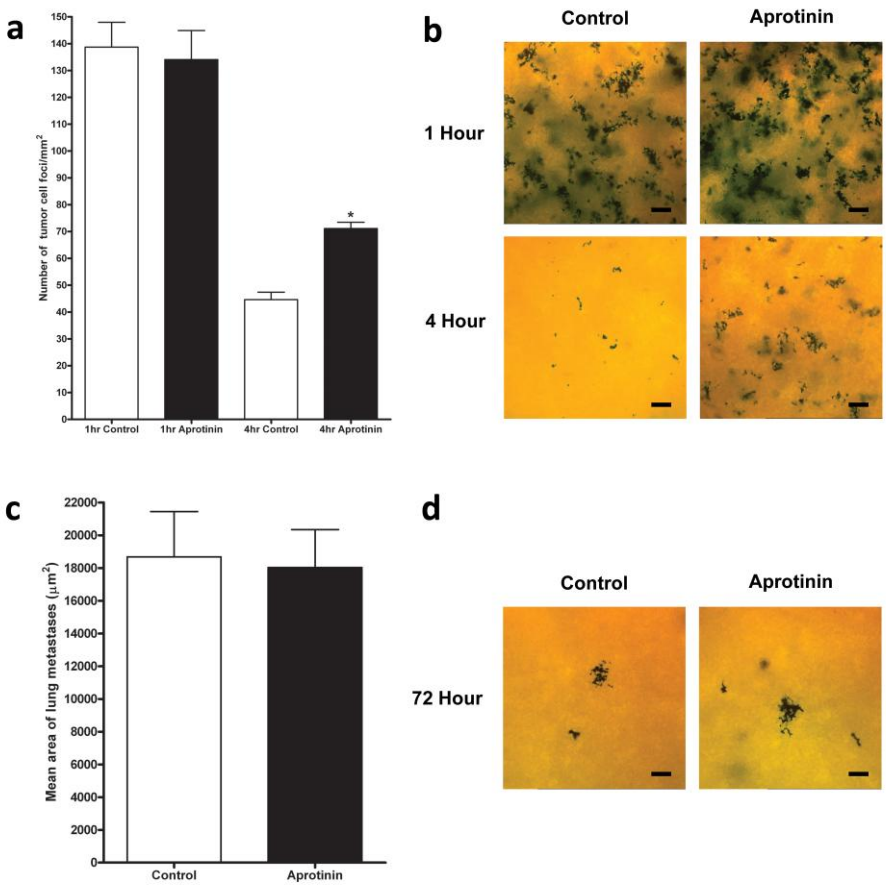

Figure 2.2 Aprotinin treatment increases the number of B16F10-LacZ cell foci at early time points.

Animals pretreated with saline or aprotinin received B16F10-LacZ cells via the lateral tail vein were sacrificed at 1 and 4 hours following cell injection (n = 8/group/time point). a) Aprotinin treatment increased number of tumor cell foci visualized 4 hours following injection (t-test, $p < 0.001$). Columns, mean; bars, SE. b) Representative images of LacZ expressing tumor cell foci in whole lung 1 or 4 hours following cell injection. Aprotinin treatment did not affect initial growth of metastases. c) Mean area of lung surface metastases 72 hours following tumor cell injection. Columns, mean; bars, SE. d) Representative images of LacZ expressing tumor cell foci in whole lung 72 hours following cell injection. Scale 50 μm.

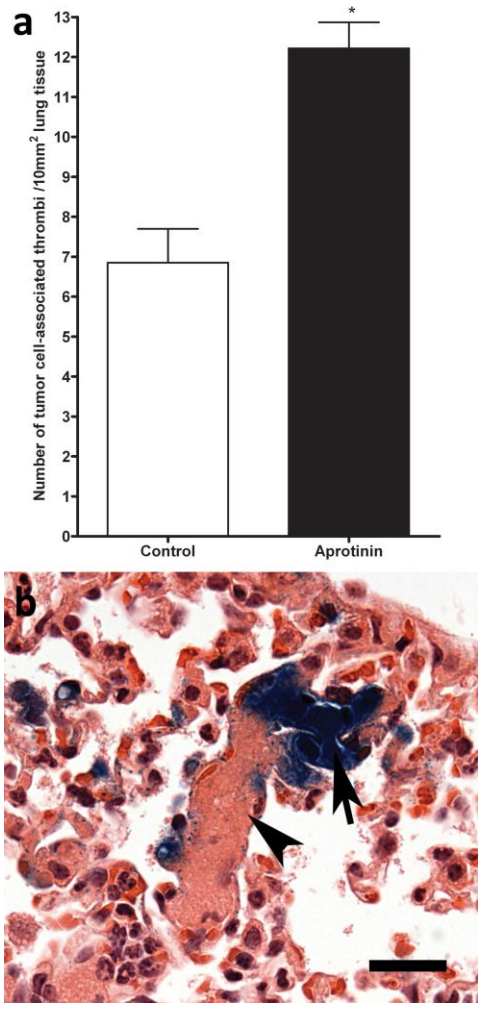

Figure 2.3 Aprotinin treatment increased association between thrombi and B16F10-LacZ cells.

a) Quantification of the number of X-gal stained tumor cells found in association with thrombi in the lung (t-test, $p < 0.05$). Animals were pretreated with saline or KIU aprotinin prior to receiving B16F10-LacZ cells (n = 8/group). Mice were sacrificed 1 hour following cell injection and lungs were stained to identify β-galactosidase expressing cells. H&E sections through all 5 lung lobes were examined to determine the number of B16F10-LacZ cells in association with thrombi. Columns, mean; bars, SE. b) Representative H&E sections showing X-gal stained B16F10-LacZ cells (arrows) in association with thrombi (arrowheads). Scale 20 μm.

following cell injection. A significant increase in the size of thrombi surrounding tumor cells was identified in the aprotinin-treated animals (t-test, $p < 0.005$, Figure 2.5). All tumor cells scanned had an associated thrombus at 4 hours in control and aprotinin-treated mice, indicating that aprotinin does not appear to affect initiation of thrombus formation. However, aprotinin functions to maintain the tumor cell-associated thrombus, presumably due to inhibition of plasmin-mediated fibrinolysis, resulting in significantly larger thrombi surrounding the tumor cells 4 hours after cell injection.

2.4.6 Plasmin-specific inhibition increases lung metastasis

The above data support the idea that the aprotinin-mediated increase in lung metastasis is due to increased association of B16F10-LacZ cells with microthrombi in the lung vasculature leading to increased survival or retention of tumor cells in the lung. To determine if the increased association between tumor cells and thrombi is due to inhibition of plasmin, animals treated with the plasmin-specific inhibitor EACA were compared to saline- and aprotinin-treated animals. Animals were treated with EACA, aprotinin or saline prior to tumor cell injection and the number of lung surface metastases present at 14 days was quantified. Specific inhibition of plasmin by EACA was found to significantly increase the number of B16F10-LacZ lung surface metastases as compared to control (t-test, $p < 0.05$, Figure 2.6). The increase in metastasis following EACA treatment was equivalent to the increase seen in the aprotinin-treated group (Figure 6), indicating that the majority of the effect seen with aprotinin is due to plasmin inhibition.

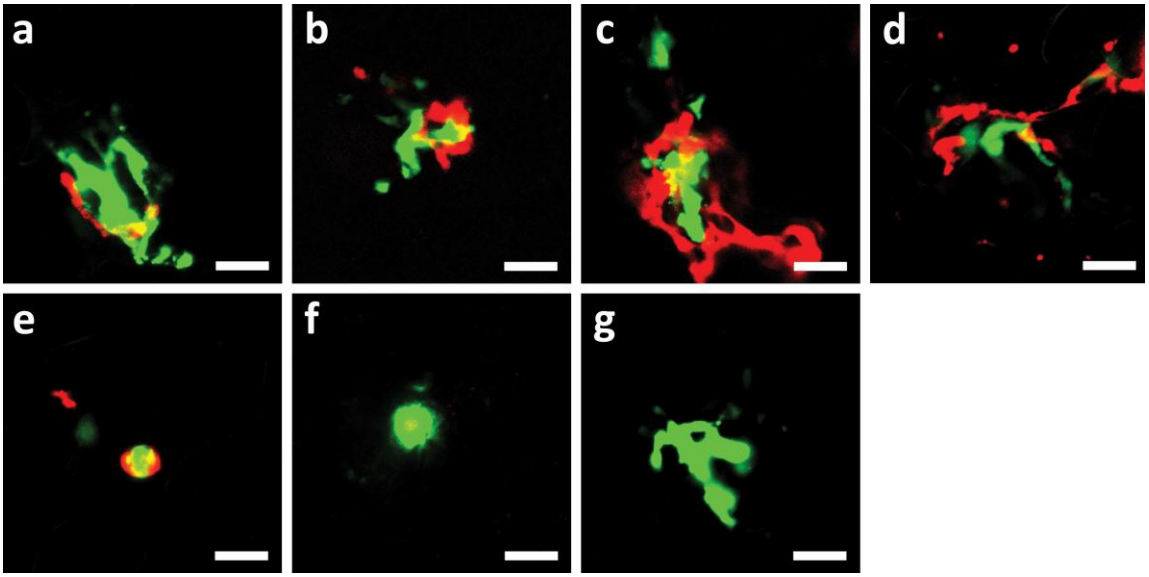

Figure 2.4 Analysis of the dynamic association between B16F10-LacZ cells and thrombi using confocal microscopy.

Tumor cells were treated in vitro with CMFDA to provide green fluorescence. Animals received AlexaFluor647 conjugated fibrinogen prior to injection of fluorescent B16F10-LacZ cells to allow incorporation of fluorescent fibrin into thrombi surrounding tumor cells. Animals were sacrificed at a) 5 minutes b) 2 hours c) 4 hours d) 6 hours and e) 24 hours following cell injection. f) Animals received a bolus injection of 10μm inert fluorescent microspheres instead of tumor cells and were sacrificed and perfused with HBSS 1 hour later. No association between fluorescent fibrin(ogen) and microspheres was identified. g) Animals treated with heparin prior to tumor cell injection showed no association between tumor cells and fluorescent fibrin(ogen). Images are representative of the mean thrombus to tumor cell ratio at each time point. Scale 20 μm.

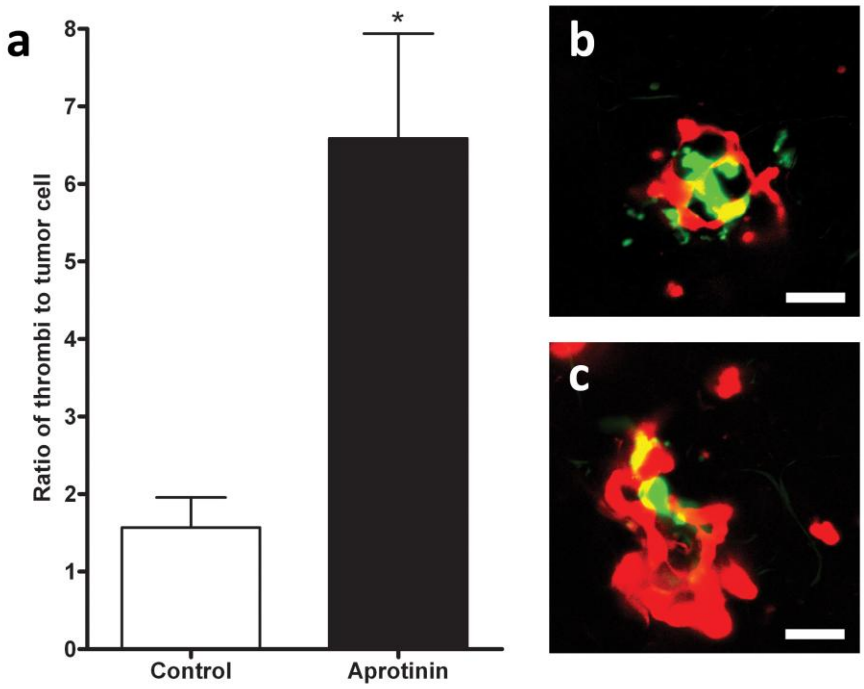

Figure 2.5 Aprotinin treatment increased association between B16F10-LacZ cells and fluorescent thrombi.

a) Quantification of confocal microscopy images taken from saline and aprotinin treated animals (n = 9/group), (t-test, $p < 0.005$). Images are expressed as a ratio of red:green, or amount of thrombus to amount of tumor cell present. Columns, mean; bars, SE. Representative confocal images taken from (b) control and (c) aprotinin treated animals.

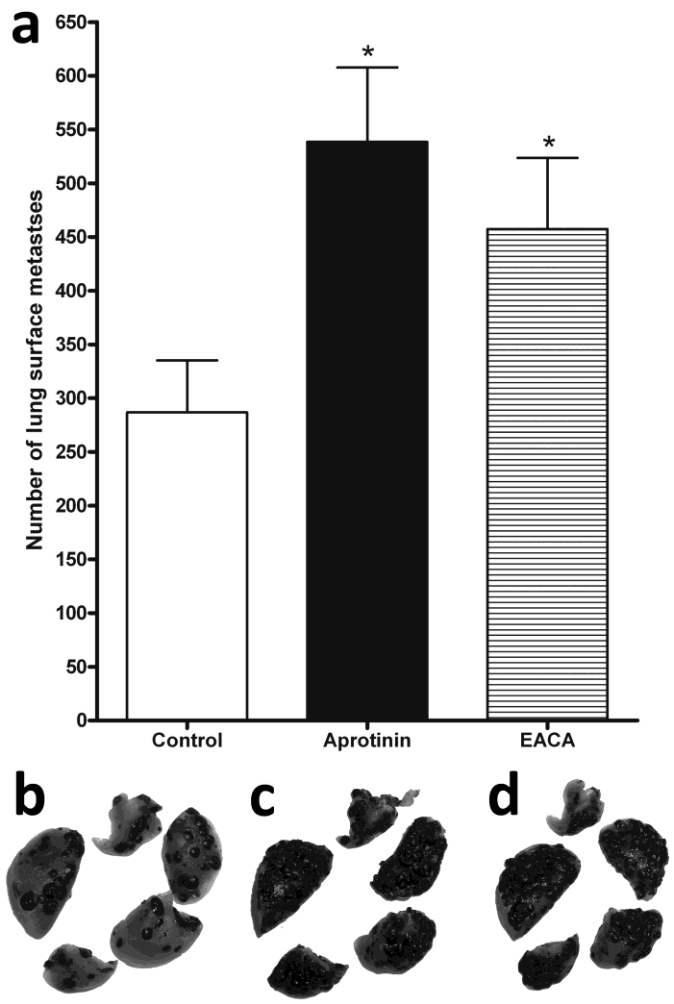

Figure 2.6 Plasmin-specific inhibition gives an equivalent increase in lung surface metastases as aprotinin treatment.

a) Animals were treated with EACA, aprotinin or saline prior to injection of B16F10-LacZ cells via the lateral tail vein (t-test, $p < 0.05$). Animals were sacrificed 14 days following cell injection and the number of lung metastases was quantified. Columns, mean; bars, SE. Representative images of whole lungs from (b) control (c) aprotinin treated and (d) EACA treated animals.

2.5 Discussion

The effect of anti-fibrinolytic agents on metastasis has not been well established to date. In particular, the study of single tumor cells interacting with thrombi in the microvasculature has not been examined, likely due to technical limitations. In the present study, a well-established model of experimental metastasis was used to investigate the effect of anti-fibrinolytic therapy on metastasis. Lung metastases were established by direct injection of B16F10-LacZ into the lateral tail vein of syngeneic mice. This injection route results in pulmonary metastasis, as the first capillary bed cells encounter from this injection site is the lung[36, 37]. Treatment of mice with aprotinin or EACA prior to injection of B16F10 tumor cells mimics the clinical setting in which patients receive anti-fibrinolytic therapy prior to surgery or tumor resection[1]. Tumor manipulation during surgery is thought to release a bolus of tumor cells into the circulation[39], a process which is mimicked by a bolus intravenous injection of cells in the experimental model. This model allows quantification of early time points following tumor cell introduction and arrest in the lung.

It was determined that anti-fibrinolytic treatment increased the total number of lung metastases formed from B16F10-LacZ cells. This increase was not due to a direct effect on the tumor cells themselves, as in vitro treatment of cells with aprotinin did not affect their in vitro growth patterns or their metastatic ability in vivo. It is well-established that B16F10 cells induce local coagulation upon arrest in secondary organs[40-43] and this association has been shown to increase metastatic burden of several types of tumor cells, including melanoma[17, 18, 44, 45]. Despite an increase in tumor cell-associated thrombi, aprotinin did not appear to affect initial cell arrest, as there was no difference in the number of tumor cell foci in the lung one hour following cell injection. However, aprotinin increased cell survival or retention in the lung, as significantly more tumor cells were present in aprotinin-treated animals four hours following cell injection. This increase may be due to a persistent increase in tumor cell association with thrombi present in large vessels at 1 hour as determined by histology, and in the microvasculature, as identified by our technique, leading to increased cell survival at 4 hours.

To evaluate the effect of aprotinin on thrombus-tumor cell association, a novel and simple confocal microscopy technique was developed here, to evaluate tumor cell-associated thrombi at early points during metastasis. This novel imaging technique allowed for the first direct investigation of the effect of anti-fibrinolytic therapy on individual tumor cell interaction with the microvasculature. Fluorescently labeled fibrinogen, injected prior to tumor cell delivery, produced clearly visible thrombi in the microvasculature that was specifically induced by the local presence of tumor cells. Little or no fluorescent signal was visible in areas devoid of tumor cells, or in areas of physical microvasculature blockage by inert microspheres. This technique offers advantages over previously reported techniques utilizing fluorescently labeled antibodies to fibrinogen or platelets as these techniques may label only the surface of thrombi and require a specialized chamber to maintain perfusion and ventilation of the lung *ex vivo*[16]. The use of this confocal microscopy technique will facilitate future studies of interactions of tumor cells with thrombi, and enables investigation of the dynamic interaction of tumor cells with thrombi, by identifying only those thrombi that have formed following injection of fluorescent fibrin(ogen). Additionally, the simplicity of this new technique allows for the study of coagulation in other areas of research such as sepsis, atherosclerosis, Crohn's disease and others, without requiring extensive protocol development or equipment.

To determine if the increase in metastasis seen in aprotinin-treated animals was due to reduced clot dissolution caused by inhibition of plasmin, animals were treated with the plasmin-specific inhibitor EACA. Treatment with aprotinin and EACA gave an equivalent increase in the total number of lung surface metastases following injection of B16F10-LacZ cells.

Previous work has shown that aprotinin may potentially inhibit metastasis at several steps[46, 47], including reduction of cell intra- and extravasation, decreased growth factor activation and inhibition of angiogenesis[14]. Conversely, the increase in metastasis in animals with a predisposition to thrombosis indicates that the extended presence of thrombi may increase metastasis. Additionally, the observed reduction in metastasis following treatment with the anti-coagulant, heparin indicates that association between

B16F10-LacZ tumor cells and thrombi is essential for lung metastasis, as has been observed in other tumor models[48-50]. This reduction in metastasis following anti-coagulant therapy is mimicked in mice with a FVIII deficiency[26], indicating that stabilization of thrombi by anti-fibrinolytic agents may result in an increase in metastasis, as was identified in this study. Treatment with anti-coagulants has also been shown to reduce tumor cell adhesion to the vascular endothelium by interfering with tumor cell-Selectin binding, and has been found to alter the distribution of tumor cells that have been directly injected, giving rise to extra-pulmonary metastases following tail vein injection (reviewed in [48]). Additionally, treatment of patients with advanced malignancy with low molecular weight heparins was found to improve patient survival[51, 52], especially in those patients with a more favorable starting prognosis[52].

The interaction between specific cancer cell types and coagulation will have varying degrees of importance, depending on the degree of coagulation activation. Therefore metastasis those cancer cells capable of causing extensive coagulation will be affected more drastically by both anti-coagulant and anti-fibrinolytic agents. This complex interplay between metastatic cells and the host coagulation system indicates that the importance of the interaction may be specific to certain tumor cell types and that the timing of anti-coagulant or anti-fibrinolytic therapies may become important during cancer treatment, with the majority of the effect of these agents occurring while tumor cells remain in the vasculature.

Interaction between hemostatic factors during hematogenous metastasis has been known for many years. Patients with metastatic disease often have complications resulting from a hyper-coagulable state, including deep vein thrombosis and disseminated intravascular coagulation[53]. Many tumor cells activate coagulation upon their arrest in the vasculature by expressing pro-coagulant molecules such as TF or CP[18, 19]. The association of tumor cells with thrombi in the vasculature has been shown to increase the metastatic ability of tumor cells, by increasing cell spreading along the endothelium[16], increasing retention of tumor cells in the secondary tissue[16, 18, 34, 54], and by increasing tumor cell survival[17, 54, 55]. Specifically, the interaction of tumor cells with activated platelets is important for metastasis, as mice lacking platelets or possessing platelets that cannot be activated by

thrombin both show marked reduction in metastasis[41, 54]. It has also been demonstrated that tumor cells and platelets can cooperate to induce increased endothelial cell retraction, allowing for extravasation of LLC cells[56]. Additionally, platelets have been proposed to increase tumor cell proliferation and enhance tumor cell interaction with the extracellular matrix[57].

Fibrin(ogen) is also associated with increased metastasis, as fibrinogen knockout mice show reduced spontaneous and experimental metastasis of LLC cells[17, 54]. Circulating fibrin(ogen) has also been found to be essential for effective colonization of the mouse lung by preventing lysis of tumor cells by NK immune cells[17, 43, 54]. This function is shared by circulating platelets, indicating that thrombus formation leads to a reduction of NK cell-mediated killing of tumor cells. Given the substantial benefit of micro-thrombi to tumor cells in the vasculature, stabilization of these thrombi by aprotinin-mediated inhibition of plasmin activity could significantly increase tumor cell survival.

Following publication of several observational studies that showed an increase in mortality following aprotinin treatment[6, 7], aprotinin is no longer utilized during cardiac surgery. Instead, the lysine analogs TXA and EACA have begun to be used clinically to reduce blood loss. A review of clinical data found that the currently used dosing regimens of TXA and EACA do not provide an equivalent reduction in blood loss as is seen with aprotinin treatment[1]. As a suitable alternative to aprotinin to reduce blood loss during surgery is considered, treatment of patients undergoing cancer-related surgeries with an anti-fibrinolytic agent requires careful evaluation, given the significant increase in metastasis observed in the present study following aprotinin and EACA treatment.

With clinical use of aprotinin suspended, this report highlights the need to evaluate novel anti-fibrinolytic agents to determine their effect on tumor cell induced thrombi and tumor cell survival prior to their widespread use in the clinical setting. The experimental metastasis model and the novel method of evaluating tumor cell–thrombus interactions at the single cell level described in this report will prove invaluable in this regard.

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Chapter 3

3 Primary melanoma tumor inhibits metastasis through alterations in systemic hemostasis

3.1 Synopsis

Progression from a primary tumor to the formation of distant metastases requires extensive interactions between individual tumor cells and their microenvironment. Importantly, the primary tumor is not only the source of metastatic cells shed into the circulation, but it can also modulate the host response to these cells, leading to an enhancement or inhibition in metastatic progression. Primary tumor-mediated stimulation of host bone marrow can result in pre-metastatic niche formation and increased metastasis, but a primary tumor can also inhibit metastasis through concomitant tumor resistance – inhibition of tumor growth by existing tumor mass. Herein, the presence of a B16F10 primary tumor was found to significantly restrict the number and size of experimental lung metastases through reduction of circulating platelets, which limited the formation of metastatic tumor cell-associated thrombi. Tumor-bearing mice displayed splenomegaly that correlated with primary tumor size and platelet count. The reduction in platelet number in primary tumor-bearing animals was specifically responsible for metastatic restriction, as restoration of platelet numbers using isolated murine platelets re-established both tumor cell-associated thrombus formation and experimental metastasis to levels seen in tumor-naïve animals. The consumption of platelets due to the presence of a B16F10 primary tumor is thought to be a form of concomitant tumor resistance and demonstrates the extensive impact a growing tumor can have on a host beyond the local environment. Understanding the interplay between a primary tumor and secondary metastases is essential, as further identification of mechanisms behind concomitant tumor resistance may allow inhibition of metastatic growth following clinical tumor resection.

3.2 Introduction

The evolution of metastatic disease from a primary tumor occurs though a series of essential steps – tumor cell invasion and intravasation, arrest in a distant capillary bed, extravasation and growth initiation in the secondary organ, and sustained growth with recruitment of a new vasculature[1]. Survival of metastatic cells during each of these steps depends on interaction with and evasion of host responses to both the primary tumor and metastatic cells[2]. The interaction between a primary tumor and the development of distant metastases has long been acknowledged, with clinical evidence of massive metastatic outgrowth following removal of primary breast and colon carcinomas[3-5]. The mechanism behind concomitant tumor resistance, the ability of a primary tumor to restrict the development of a second tumor[6, 7], has not been resolved. Better understanding of concomitant tumor resistance would allow metastatic inhibition through supplementation or depletion of factors to mimic the presence of a primary tumor, and thereby continue secondary tumor growth restriction after primary tumor resection.

Three hypotheses have been proposed to explain the protection from metastatic growth by a primary tumor: concomitant immunity, whereby a primary tumor may prime the host immune system and enhance clearance of metastatic cells[8]; production of inhibitory factors (anti-mitogenic or anti-angiogenic) from the primary tumor which prevent growth of the secondary tumors[5, 9]; and athrepsia, the depletion of required systemic factors by the primary tumor thereby preventing metastatic establishment (reviewed in [7]). Investigation of concomitant tumor resistance led to the ground-breaking discovery of angiostatin, an anti-angiogenic factor produced by a primary tumor that is capable of preventing angiogenesis in secondary tumors[10]. Concomitant immunity has been found to occur only in highly immunogenic tumor types, such as those arising following chemical insult, and only while those tumors remained small (<500 mm^3)[11], unless animals were depleted of specific regulatory T cells[12]. The concept of athrepsia as it applies to metastatic growth has not been well investigated to date[7].

It is well established that coagulation factors are essential to successful metastasis formation[13], especially for B16 murine melanoma cell lines[14-16]. Our group and others have shown that elimination of individual coagulation factors restricts pulmonary

metastasis formation[14, 17-20], in part by allowing increased NK immune cell killing of tumor cells[16, 21, 22]. Stabilization of cell surface thrombi using the serine protease inhibitor aprotinin increased metastasis formation due to sustained interaction between thrombi and metastatic B16F10 cells[14]. B16F10 cells express TF and are individually capable of activating the coagulation cascade[23]. Thus it was hypothesized that cells within a B16F10 primary tumor may stimulate thrombus formation and may deplete systemic coagulation factors such as platelets. Platelets have long been hypothesized to support cancer progression[24] and have been definitively linked to increasing formation of metastases through stabilization of tumor cell arrest and increasing survival in the vasculature, as well as increasing extravasation and proliferation in secondary tissues.[16, 25] Given that tumor cell-associated thrombi are essential for successful metastatic progression, we hypothesized that the presence of a B16F10 primary tumor would therefore restrict lung metastasis.

Here we analyzed the effect of a B16F10 primary tumor on metastasis formation. We found a significant inhibition of lung metastasis formation following a secondary intravenous injection of B16F10-LacZ cells in primary tumor-bearing mice. This decrease was due to reduced thrombus formation at the metastatic site (lung) caused by a significant reduction in circulating platelet numbers. Re-constitution of platelet counts in tumor bearing mice using isolated murine platelets restored cell surface thrombi and led to equivalent metastasis formation in tumor-bearing and tumor-naïve mice.

3.3 Materials and Methods

3.3.1 Cell Culture

B16F10 and B16F10-LacZ[14] murine melanoma cells (American Type Culture Collection, Manassas, VA) were maintained in α-minimal essential media (Invitrogen, Burlington, Canada), supplemented with 10% fetal bovine serum (Sigma, Mississauga, Canada) and 1 mg/ml hygromycin (Invitrogen, B16F10-LacZ only). For injection into mice, cells were trypsinized from 70-80% confluent plates and suspended in cold, sterile Hanks Balanced Salt Solution (HBSS, Invitrogen) to a final concentration of 5×10^6/mL.

3.3.2 Experimental Metastasis Model

Female C57Bl/6 mice (6-7 weeks old, Charles River) were lightly anesthetized with isofluorane and 100 μl of the B16F10 cell preparation (primary tumor group) or cell vehicle (HBSS, tumor-naïve group) was injected orthotopically into the dermis (i.d.) on the right hind flank. Tumor growth was evaluated by measurement with calipers in two perpendicular dimensions and the tumor volume was estimated using the following formula volume = 0.52 $(width)^2(length)$, for approximating the volume (mm^3) of a ellipsoid. Primary tumors were allowed to grow for 16 days, at which time B16F10-LacZ cells were trypsinized from 70-80% confluent plates and suspended in cold, sterile HBSS. Unanesthetized tumor-bearing and tumor-naïve mice then received an intravenous (i.v.) injection of $5x10^5$ cells in 100 μl HBSS via the lateral tail vein. Animals were sacrificed 6 days following i.v. injection and lung tissue was isolated, stained for β-galactosidase activity and the number of lung surface metastases quantified.

For platelet restoration studies, 100 μl of the platelet suspension (see 3.3.5 Platelet isolation and preparation) or vehicle was injected via the lateral tail vein 5 minutes prior to i.v. B16F10-LacZ cell injection. Animals were sacrificed 6 days following i.v. injection and lung tissue was isolated, stained for β-galactosidase activity and the number of lung surface metastases quantified.

All mice were 7 - 8 weeks of age at the time of injection and were cared for in accordance to the Canadian Council on Animal Care, under a protocol approved by the University of Western Ontario Council on Animal Care.

3.3.3 Quantification of lung metastases

To enable visualization of lung metastases, mice were euthanized 6-days following i.v. cell injection and whole lungs placed in phosphate buffer (0.1 M sodium phosphate monobasic, 0.1 M sodium phosphate dibasic, pH 7.3), on ice. Lungs were stained with X-gal (Bioshop, Burlington, Canada) solution as described [26] to visualize LacZ-expressing cells. The total number of surface-visible metastases was determined on intact lung lobes using a stereomicroscope. Area of individual lung metastases was determined using the Axiovert 200M microscope and Axiocam HRC camera utilizing

Axiovision 4.6 software. Linear length and width of each metastasis was determined, and area of each was estimated by assuming an approximately elliptical shape and using the formula π x length x width/4.

3.3.4 Assessment of circulating platelet numbers

Sixteen days after hind flank injection of cell vehicle or B16F10 cells, tumor-naïve and B16F10 primary tumor-bearing mice were anesthetized with isoflurane and blood was drawn by cardiac puncture using a heparinized syringe and collected into purple top (ethylenediaminetetraacetic acid, EDTA) 2 mL tubes (BD Biosciences, Mississauga, Canada). The number of platelets was determined on a Coulter LH 780 Hematology analyzer. The normal platelet count seen in mice is 900 – 1600 x 10^3 / µl but the number quantified on clinical hematology analyzers is frequently underestimated due to the smaller size of murine platelets.[27] To ensure this did not skew our data, all quantifications were performed on the same machine. For platelet re-constitution studies, mice received an i.v. injection of platelets 5 minutes prior to cardiac puncture and blood collection as above to determine the number of platelets present when B16F10-LacZ cells would be delivered.

3.3.5 Platelet isolation and preparation

For platelet re-constitution experiments, blood was obtained from female C57Bl/6 retired breeders (30 - 35 g) under isofluorane anaesthetic via cardiac puncture using citrated 3 mL syringes. Blood was collected into 2 mL Eppendorf tubes with citrate-dextrose solution (0.1 M trisodium citrate, 0.11 M dextrose, 71 mM citric acid monohydrate) at 1:9 citrate-dextrose:blood ratio. Platelets were isolated using a differential centrifugation protocol based on Musaji et al.[28]. Briefly, whole blood was centrifuged at 220 g for 6 minutes at 4°C and platelet rich (PR) upper layer was collected. Cold, buffered saline glucose citrate (BSGC: 8.6 mM Na_2HPO_4, 1.6 mM KH_2PO_4, 0.12 M NaCl, 0.9 mM EDTA, 13.6 mM NaCitrate, 11.1 mM D-Glucose, pH 7.3) was added to each tube to 1.75 mL and inverted to mix. PR upper layer was collected following another 220 g spin for 6 minutes at 4°C. This step was repeated once more. PR suspensions were pooled and centrifuged at 1613 g for 15 minutes at 4°C to pellet platelets. Supernatant was discarded

and platelets were resuspended in 1 mL cold BSGC. To isolate platelets from other cellular components, the platelet suspension was centrifuged at 220 g for 6 minutes at 4°C and PR suspension was transferred to a clean Eppendorf tube. Platelets were pelleted at 1613 g for 10 minutes at 4°C and supernatant discarded. Platelets were then resuspended to appropriate volume in BSGC for immediate injection. To confirm that platelets had not become activated during the isolation procedure, a small aliquot was removed from the final suspension to be tested. Only after the addition of thrombin did the platelets clump and the previously cloudy suspension become clear. Animals received a 100 µl i.v. injection that contained platelets concentrated from 3 or 6 mL of whole murine blood. Only those vials that showed no evidence of coagulation were used for platelet isolation.

3.3.6 Analysis of coagulation using confocal microscopy

To enable analysis of coagulation in vivo, confocal microscopy was performed using a previously developed technique[14]. B16F10-LacZ cells were labeled in vitro using 5-chloromethylfluorescein diacetate, Cell Tracker Green (CMFDA, Molecular Probes, Burlington, Ontario, Canada), according to the manufacturer's instructions. Ten minutes before cell injection, animals received intravenous injection of 0.12 mg AlexaFluor647-conjugated fibrinogen (Molecular Probes) in 80 µl sodium bicarbonate, pH 8.3. Four-hours following cell injection, animals were euthanized by an intraperitoneal injection of xylazine/ketamine (5.2 mg ketamine and 0.26 mg xylazine per 20 g body mass). The lung was cleared of blood by gravity perfusion of HBSS through the right ventricle of the heart. 'Flow through' perfusate left the body via a puncture made in the left atrium. Lungs were perfused for 2 minutes, excised and frozen at –80°C to allow for later analysis by confocal microscopy. To visualize, lungs were thawed in cold HBSS prior to transfer to 0.1% paraphenylenediamine in HBSS for 10 minutes. Lungs were assessed using a Zeiss LSM 410 confocal microscope, and Carl Zeiss LSM 3.99 software. The presence of tumor cells was determined using green fluorescence and the focal plane containing the largest volume of tumor cells was identified. At that same focal plane, the lung then was scanned for red fluorescence to identify thrombi. The green and red fluorescence were quantified in pixels by a blinded observer using ImageJ[29], and the ratio

of thrombus to tumor cell area was determined. To ensure that visualized red fluorescence was due to thrombus formation and not accumulation of fluorescent fibrinogen that was not cleared, potentially due to vessel blockage by tumor cells, inert plastic microspheres (20 μm, Polysciences, Pennsylvania) were injected. Use of these microspheres in platelet-restoration experiments allowed examination of lung tissue for non-specific thrombus formation following platelet injection.

3.3.7 Histology

Spleens were collected and weighed; long bones were stripped of muscle tissue prior to fixation of tissues in 10% buffered formalin. Bones were decalcified in Cal-Ex II (Fischer Scientific, New Jersey) solution prior to H&E staining of 3 μm sections. Megakaryocyte quantification was carried out by a trained observer under pathologist guidance.

3.3.8 Statistical analysis

The Student's unpaired t test and one-way ANOVA with a Tukey's Multiple Comparison post-test were used to compare between animal groups. The Pearson's rank correlation was used to determine relationships between various variables. All statistics were calculated using GraphPad Prism 4. A two-sided $p < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Primary tumor presence decreases metastasis

To evaluate the effect of a primary tumor on early steps in the development of metastases, we quantified metastases using a modified model of experimental metastasis. Animals first received an intradermal (i.d.) injection of B16F10 cells (primary tumor group) or cell vehicle (tumor-naïve group). After sixteen days, all animals received a secondary injection of B16F10-LacZ cells via the lateral tail vein to target cells directly to the lung. This experiment was repeated multiple times with similar results; representative data is presented here. Primary tumors were 533.5 ± 33.2 mm^3 at the time of secondary injection. We found that the presence of a primary tumor significantly

inhibited experimental lung metastasis, as six days following secondary injection there were fewer X-gal positive lung surface metastases in primary tumor-bearing mice as compared to tumor-naïve animals (Figure 3.1 A). Very few lung metastases were not positive for X-gal (3 metastases in all mice examined), and these tumors were significantly larger than the other metastases visualized. It was assumed that these rare metastases arose from seeding by the primary tumor and were not included in subsequent quantification. X-gal positive metastases that did form in the primary tumor group were significantly smaller than those in the tumor-naïve group (Figure 3.1 B), indicating that the primary tumor is not only impeding survival of arrested cells, but also the growth of cells that do persist.

3.4.2 Primary tumor decreases thrombus formation following metastatic cell arrest

Previous work in our and other laboratories[14, 17, 18] has shown that efficient metastasis of B16F10 cells is dependent on thrombus formation at the cell surface following arrest in the lung microvasculature. Therefore, we evaluated the ability of B16F10-LacZ cells to stimulate thrombus formation following arrest in the lung of tumor-bearing and tumor-naïve animals. Using a previously developed confocal microscopy technique[14], we found that the presence of a primary tumor significantly reduced the size of thrombi that formed 4-hours after secondary cell injection as compared to tumor-naïve animals (pictured in Figure 3.2 A and B, quantified in Figure 3.2 C).

3.4.3 Primary tumor causes systemic changes including decreased platelet number and splenomegaly

To determine the mechanism by which the presence of a primary tumor inhibited thrombus formation around i.v. injected cells, the number of circulating platelets was quantified in tumor-naïve and tumor-bearing animals. Sixteen days following primary injection, blood was pulled via cardiac puncture for analysis. It was found that primary tumor-bearing mice had significantly fewer circulating platelets than tumor-naïve animals (Figure 3.3 A). Primary tumor-bearing mice were also found to have significantly enlarged spleens upon sacrifice at day 16 (Figure 3.3 B), and spleen size significantly

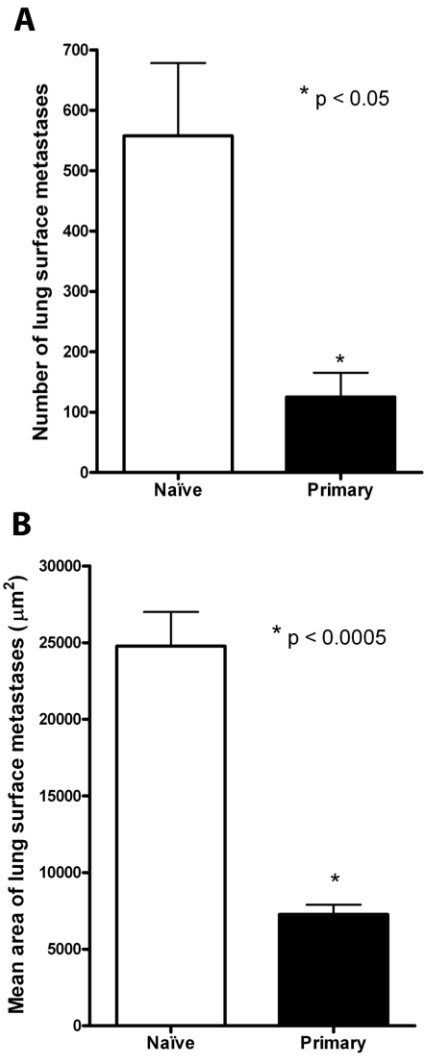


Figure 3.1 The presence of an intradermal B16F10 primary tumor significantly reduced lung metastasis from a secondary i.v. injection of B16F10-LacZ cells.

(A) Animals that received an i.d. injection of B16F10 cells had fewer lung metastases than animals that received a sham i.d. injection (n = 10-12/group; t-test $p < 0.05$). (B) Lung metastases that developed in tumor-bearing mice were significantly smaller than those in tumor-naïve animals (n = 4/group, randomly chosen from animals in (A); t-test $p < 0.005$). Columns, mean; bars, SE.

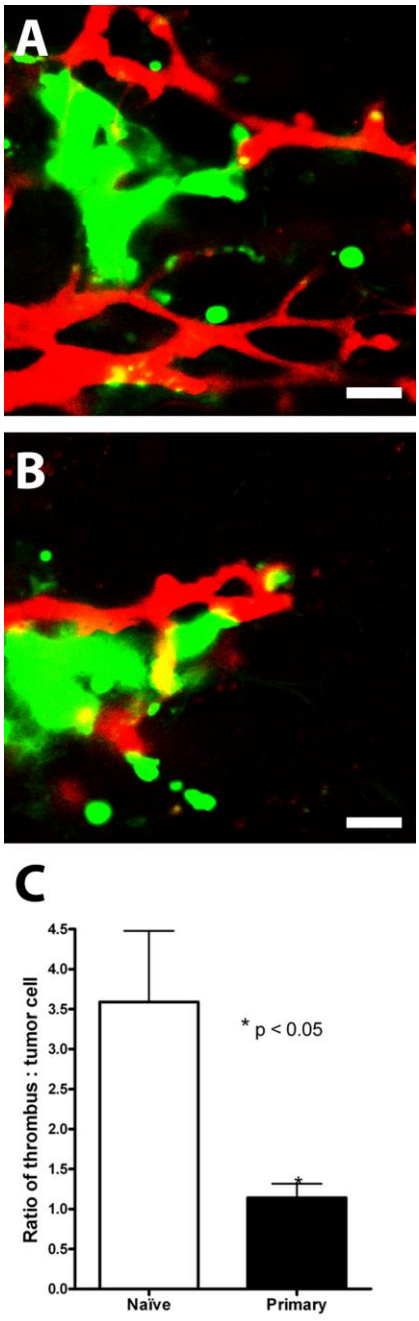


Figure 3.2 The presence of an intradermal B16F10 primary tumor reduced the association between thrombi and B16F10-LacZ cells in mouse lung microvasculature.

B16F10-LacZ tumor cells were labeled in vitro with CMFDA to provide green fluorescence. Animals received AlexaFluor647 conjugated fibrinogen prior to injection of fluorescent B16F10-LacZ cells to allow incorporation of fluorescent fibrin into thrombi. Representative confocal images taken from (A) tumor-naïve and (B) tumor-bearing animals. Scale 20 μm. (C) Quantification of confocal microscopy images taken from tumor-naïve and tumor-bearing animals (n = 4/group), (t-test, $p < 0.05$). Images are expressed as a ratio of red:green, or amount of thrombus to amount of tumor cell present. Columns, mean; bars, SE.

correlated positively with primary tumor weight (Figure 3.3 C) and negatively with platelet number (Figure 3.3 D). Tumor weight showed a trend towards a negative correlation with the number of circulating platelets (Figure 3.3 E), but this did not reach statistical significance. Histological analysis of spleens from tumor-bearing and tumor-naïve animals was performed to further assess the interplay between the primary tumor and the spleen. Normal spleen histology was observed in tumor-naïve animals (Figure 3.4 A and B), but it was found that the spleens isolated from tumor-bearing animals showed extensive extramedullary hematopoiesis, with tri-lineage precursors throughout the red pulp, distorting the splenic architecture and decreasing white pulp presence (Figure 3.4 C & D). In particular, there was an abundance of megakaryocytes and their precursors (Figure 3.4 D vs. 3.4 B). The observed splenomegaly (Figure 3.3 B) was thus at least in part due to marked extramedullary hematopoiesis, and may have been secondary to platelet consumption in tumor bearing animals.

To further investigate the effect of the primary tumor on platelet manufacture, long bones of primary tumor-bearing and tumor-naïve animals were removed and the number of megakaryocytes was quantified in histologic slides. Contrary to the spleen histology, there was no effect of the primary tumor on the number of megakaryocytes in the long bones (data not shown), indicating that the tumor-stimulated platelet manufacture is in the spleen alone, as is common during murine stress[27].

3.4.4 Re-establishment of platelet number in primary tumor-bearing mice restores thrombus formation

To determine if reduction in platelet number was functionally responsible for the decrease in lung metastasis, we strove to re-establish a normal platelet count in tumor-bearing mice. To this end, we isolated platelets from donor mice and injected them into tumor bearing animals sixteen days following primary tumor introduction. We concentrated platelets from 3 mL or 6 mL of blood prior to injection and found that platelets isolated from 6 mL of mouse blood was required to fully restore the number of circulating platelets in tumor-bearing animals (Figure 3.5 A). We next wanted to determine if this platelet injection allowed functional thrombus formation in response to

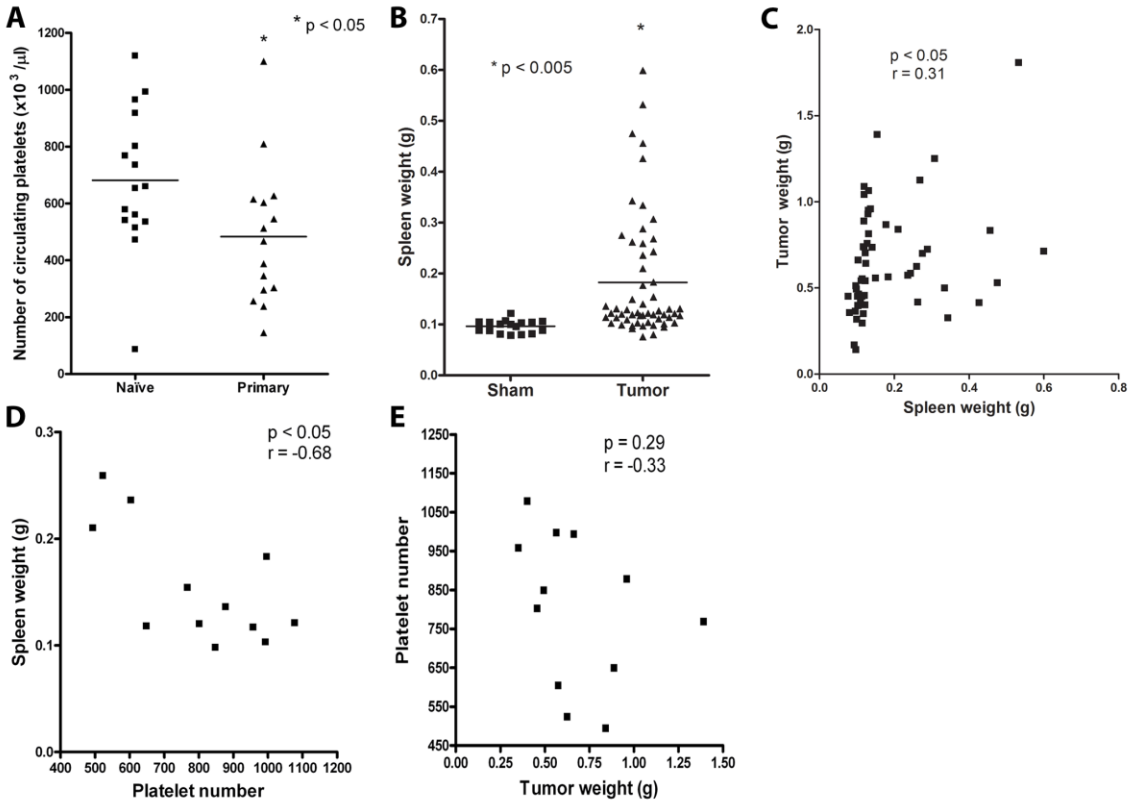


Figure 3.3 Interaction between a B16F10 primary tumor and platelet turnover.

(A) Quantification of circulating platelets in tumor-naïve and tumor-bearing animals. Animals with a B16F10 primary tumor had significantly fewer platelets sixteen days following i.d. injection (n = 12/group, t-test $p < 0.05$). (B) Tumor bearing mice were found to have significantly enlarged spleens as compared to tumor naïve animals (n= 17-56/group, t-test $p < 0.005$). Additionally, spleen weight correlated with both (C) tumor size (n = 56, Pearson's $p < 0.05$, $r = 0.31$) and (D) platelet count (n = 12, Pearson's $p < 0.05$, $r = -0.68$). (E) Correlation between tumor size and platelet count did not reach significance (n = 12, Pearson's $p = 0.29$, $r = -0.33$).

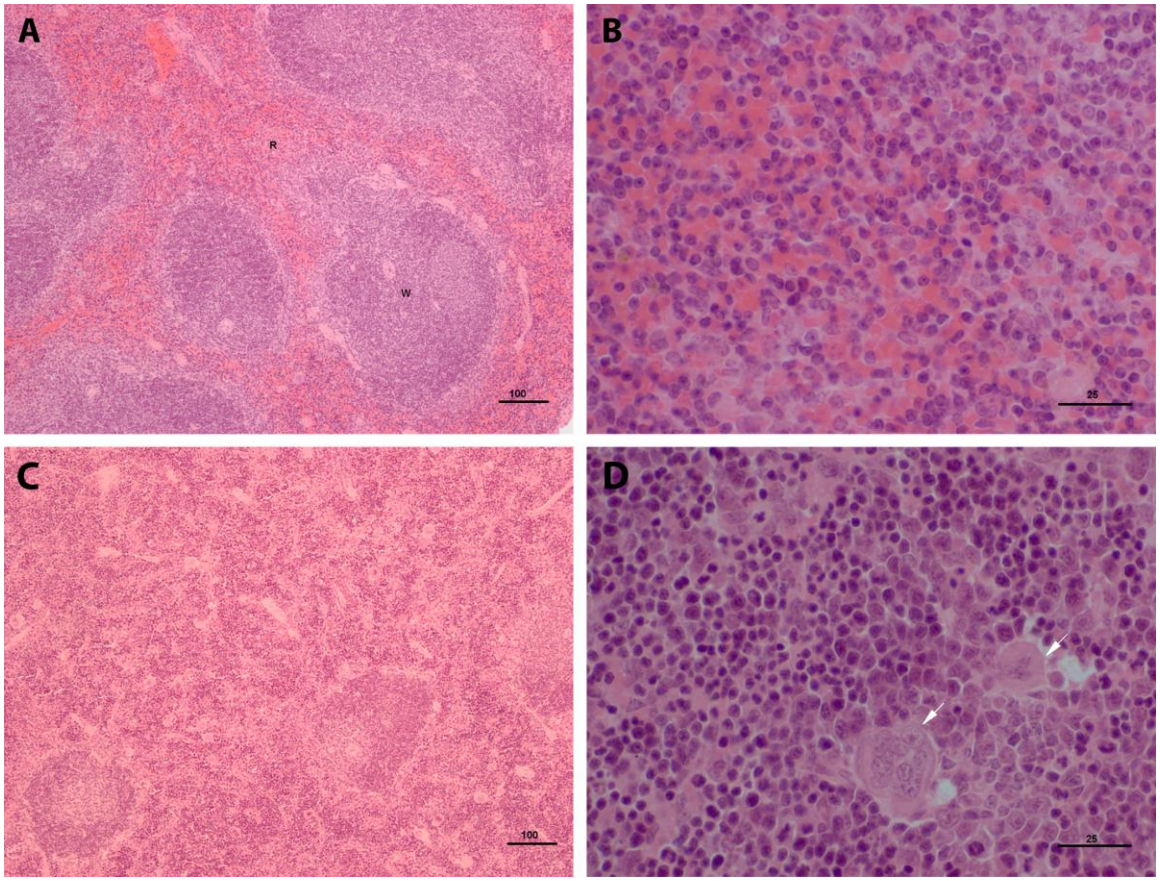


Figure 3.4 Histological analysis of splenic tissue isolated from tumor-naïve (A, B) and tumor-bearing (C, D) animals.

Normal splenic structure is seen in tumor-naïve animals, with distinct red (R) and white pulp (W). Hypercellularity is seen in the red pulp of spleens isolated from tumor-bearing animals, with reduction of white pulp. Higher power magnification shows normal red pulp in tumor-naïve spleen (B), marked tri-lineage extramedullary hematopoeisis, with a particular abundance of megakaryocytic precursors (arrows) in tumor-bearing animals (D).

secondary cell injection. Intravenous injection of AlexaFluor647-conjugated fibrinogen prior to injection of unlabeled platelets or vehicle and CMFDA-labeled tumor cells allowed visualization of all thrombi formed in response to B16F10-LacZ tumor cells. Using confocal microscopy, we evaluated thrombus formation in tumor-naïve and tumor-bearing mice following platelet vehicle injection, and tumor-bearing mice after platelet injection. Injection of platelets isolated from 6 mL of blood was capable of restoring thrombus formation in primary tumor-bearing mice to levels equivalent to tumor-naïve animals (Figure 3.5 B-E). To confirm that visualized thrombi were specific to tumor cell interaction and did not arise from injection of isolated platelets, a subset of tumor naïve animals received i.v. injection of fluorescent fibrinogen prior to injection of isolated platelets. These animals did not receive B16F10-LacZ cells; rather they received an i.v. injection of 20 μm inert fluorescent microspheres. Arrest of these spheres did not result in thrombus formation or spontaneous fluorescent fibrinogen accumulation (Figure 3.5 F). This indicates that the thrombi observed following tumor cell injection are due to interaction between B16F10-LacZ cells and circulating coagulation factors.

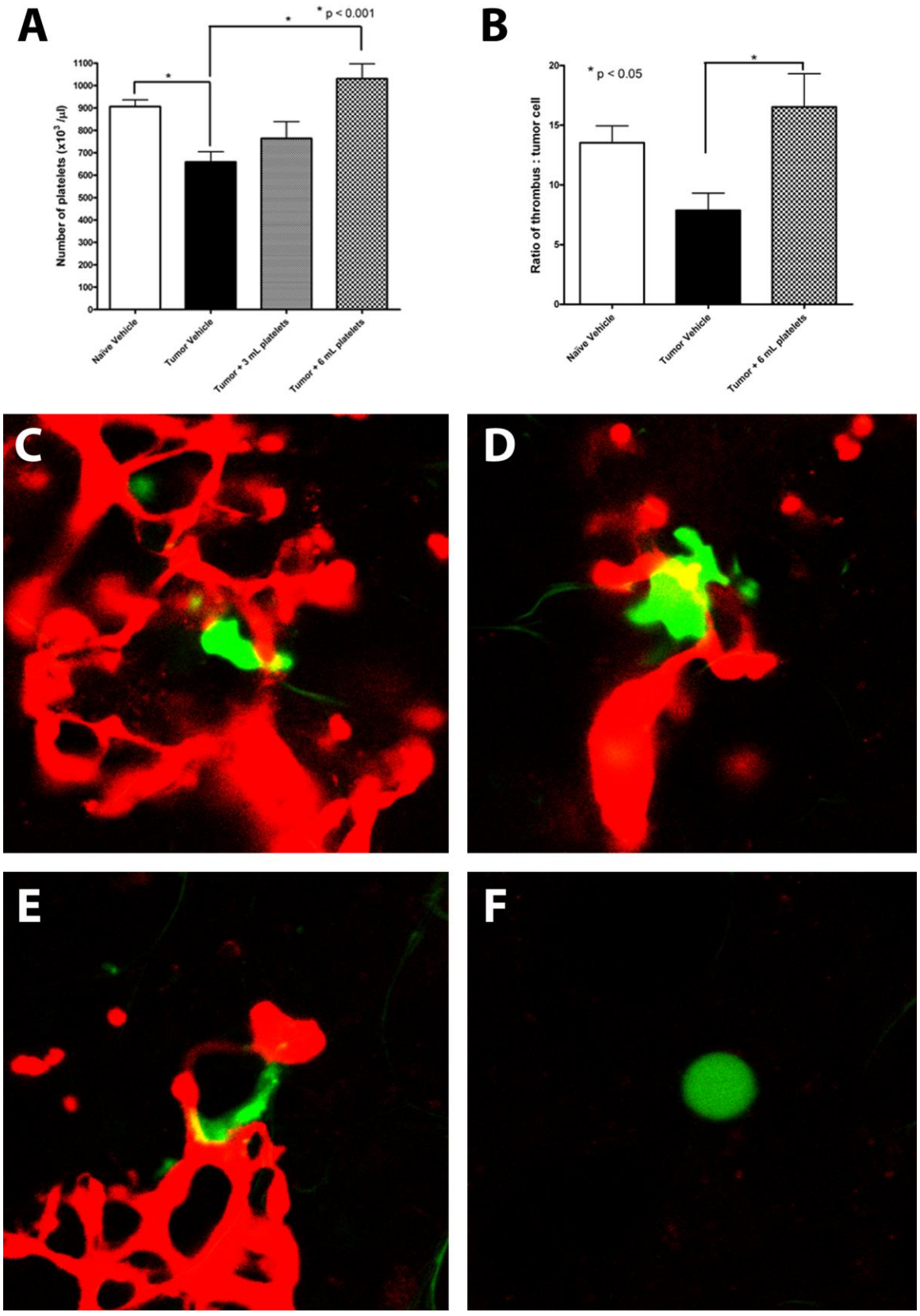
3.4.5 Normal platelet count required for B16F10 metastasis

Given that injection of platelets restored functional coagulation at the metastatic cell surface, we quantified lung metastasis formation in mice following platelet injection. Sixteen days after mice received a primary tumor or sham injection, animals received an i.v. injection of platelets or vehicle five minutes prior to i.v. injection of B16F10-LacZ cells. Six days following secondary cell injection, the number of lung surface metastases was quantified.

Tumor-naïve mice that received the platelet vehicle were found to have fewer metastases as compared to un-injected, tumor-naïve mice (Figure 3.6). This was not surprising given the anti-coagulant component of the platelet vehicle (EDTA). Importantly, tumor-bearing mice that received platelets showed more lung metastases than tumor-naïve animals that received platelet vehicle, and showed equivalent metastasis to un-injected tumor-naïve mice, indicating that the re-establishment of platelet number was capable of

Figure.3.5 Injection of isolated murine platelets re-established circulating platelet number and restores thrombotic tumor emboli.

(A) Platelets isolated from donor mice were injected into tumor-bearing mice and the number of circulating platelets was determined. Injection of platelets concentrated from 6 mL of whole blood was required to acquire platelet numbers that were not significantly different from tumor-naïve animals. Functional thrombus formation was determined using confocal microscopy. B16F10-LacZ tumor cells were labeled in vitro with CMFDA to provide green fluorescence. Animals received AlexaFluor647-conjugated fibrinogen prior to injection of fluorescent B16F10-LacZ cells, to allow incorporation of fluorescent fibrin into thrombi. (B) Quantification of confocal microscopy images taken from tumor-naïve and tumor-bearing animals following platelet vehicle injection, and tumor bearing animals following platelet injection (n = 4/group). Injection of platelets concentrated from 6 mL of whole blood restored functional thrombus formation in response to B16F10-LacZ cell injection (ANOVA, Tukey's post test, n.s. from tumor-naïve group, $p < 0.05$ from tumor vehicle group). Columns, mean; bars, SE. Representative confocal images taken from (C) tumor-naïve, (D) tumor-bearing and (E) tumor-bearing animals following platelet injection. (F) Control animals that received platelets and fluorescent fibrinogen were given an injection of inert fluorescent microspheres to test for non-specific thrombus formation. No accumulation of fibrinogen was seen. Scale bar 20 μm.


A
* p < 0.001
Number of platelets (x10³ /μl)
Naïve Vehicle
Tumor Vehicle
Tumor + 3 mL platelets
Tumor + 6 mL platelets
B
* p < 0.05
Ratio of thrombus : tumor cell
Naïve Vehicle
Tumor Vehicle
Tumor + 6 mL platelets
C
D
E
F

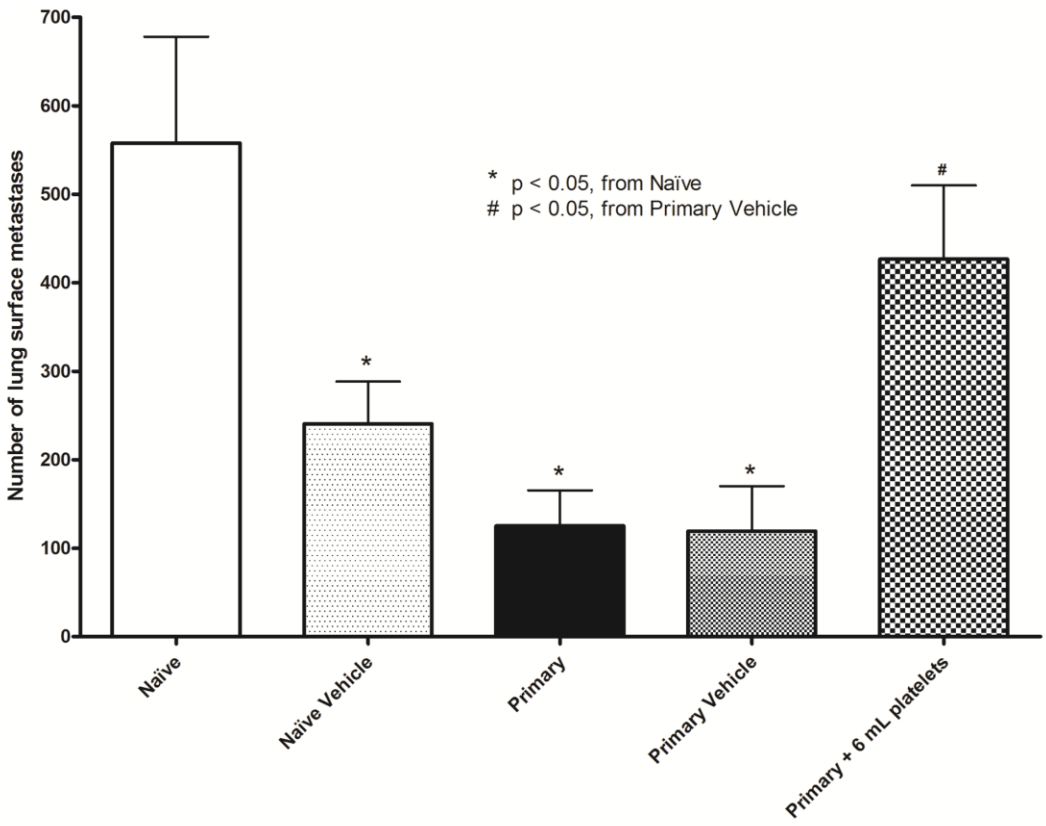


Figure 3.5 Reconstitution of platelet number in tumor-bearing animals re-established lung metastasis formation.

Tumor-naïve animals received an i.v. injection of platelet vehicle and tumor-bearing animals received an i.v. injection of platelet vehicle or isolated platelets prior to i.v. B16F10-LacZ cell injection (n = 12/group). Platelet vehicle injection into tumor-naïve animals reduced metastasis number as compared to control (un-injected naïve and primary data are historical controls from Fig 1A) (ANOVA, Tukey's post test, $p < 0.05$). Vehicle injection into tumor-bearing mice did not further reduce metastasis from that seen previously in tumor-bearing animals ($p > 0.05$). Platelet injection into tumor-bearing mice showed an increase in lung metastasis as compared to vehicle injected tumor-bearing animals ($p < 0.05$) and restored lung metastasis number to that seen in tumor-naïve animals ($p > 0.05$). Columns, mean; bars, SE.

restoring lung metastasis of B16F10-LacZ cells and was able to counteract the effect of the presence of a B16F10 primary tumor (Figure 3.6).

3.5 Discussion

In this study, we established that the presence of a B16F10 primary tumor was capable of significantly inhibiting the number and size of lung metastases arising from secondarily injected B16F10-LacZ cells. This inhibition of metastasis was found to be due to a primary-tumor mediated reduction in circulating platelet number, resulting in insufficient thrombus formation in the lung following metastatic cell injection. Restoration of circulating platelet number, through injection of platelets isolated from donor mice, re-established thrombus formation in primary tumor bearing mice and allowed equivalent metastasis formation in both primary tumor-bearing and tumor-naïve animals.

The negative impact of a B16F10 primary tumor on secondary metastasis was somewhat surprising given that the B16 cell line has been reported to stimulate pre-metastatic niche formation in the lung, liver, testis, spleen and kidney[30]. Recruitment of BMDC to facilitate formation of a pre-metastatic niche is thought to aid metastasis formation, as $VEGFR1^+$ cell present in the niche increase adhesion and arrest of metastatic tumor cells. Additionally, in vitro analysis found that B16 cells were increasingly mobile in response to $VEGFR1^+$ cells[30]. It is possible that B16F10 cells do not stimulate formation of the pre-metastatic niche as seen with the B16 cells used by Kaplan et al.[30], or that the reduction in platelet number in primary tumor-bearing animals is able to inhibit metastasis formation in spite of pre-metastatic niche presence. Development of concomitant tumor resistance has been found to occur via immunogenic (small tumors, immunogenic types) and non-immunogenic mechanisms (large tumors, anti-angiogenic or anti-mitotic mechanisms)[10, 31, 32], but the potential for a primary tumor to both inhibit and promote metastasis through concomitant tumor resistance and pre-metastatic niche formation requires further investigation. Importantly, in this study animals received the second injection of tumor cells when the primary tumor was relatively small (~530 mm^3) such that the previously identified anti-angiogenic and anti-mitotic mechanisms of concomitant tumor resistance found to occur after primary tumors reached 2000 mm^3 [11]

should not yet be involved. Additionally, immunogenic control is not thought to be responsible for the suppression of B16F10 metastases seen here, as B16 cells were derived from a spontaneously formed tumor and are poorly immunogenic[12]. Direct analysis of tumor immunity in a B16 model found that it was induction of $CD4^+$ $CD25^+$ regulatory T cells that was responsible for the lack of prevented the development of concomitant tumor immunity in C57Bl/6 mice[12].

It is well established that platelets play an integral role in metastatic establishment[33-35]. Following arrest in the vasculature, tumor cells stimulate thrombus formation at their cell surface[14, 16, 18]. The interaction between tumor cells and thrombi provides valuable adhesion contacts[36], growth and survival factors and protection from host immune surveillance[16, 34]. In pre-clinical studies, treatment of animals with an anti-coagulant such as heparin[14, 37] or hiruden[17] prior to metastatic cell injection results in significant reduction in pulmonary metastasis[38]. Previous work in our laboratory also found that inhibition of serine-protease activity in vivo stabilized tumor cell-associated thrombi and increased B16F10 metastasis formation[14]. Activated platelets were found to increase invasiveness of human ovarian cancer cells[39] and tumor cells that are able to stimulate clot formation are more likely to be retained in the pulmonary vasculature as they are more likely to form stable adhesive contacts and spread along the inside of the pulmonary vasculature[15]. Induction of thrombocytopenia through injection of bacterial lipopolysaccharide or neuraminidase significantly decreases lung metastasis of both strongly and weakly immunogenic tumor types[40]. Interestingly, injection of platelets into thrombocytopenic animals prior to i.v cell injection did not restore metastasis number, potentially due to insufficient platelet injection, as evidence for platelet number rescue was not presented[40]. The direct role of platelets in establishment of metastases has been investigated in B16 and fibrosarcoma models with conflicting results. The positive correlation between platelet aggregation and metastatic ability found in the B16F10 cell line did not hold when individual B16F10 clones were generated and tested.[41] Similar clonal investigation with the PAK 17 fibrosarcoma cell line showed that highly metastatic clones require platelet interaction for successful metastasis. This disparity could be due to differences in analysis of platelet aggregating ability[42], but could indicate that the most aggressive B16F10 clones are capable of metastasis regardless of platelet interaction, due

to other malignant characteristics. For example, those clones that exhibit high metastatic ability without extensive platelet aggregation activity may form more extensive tumor cell-tumor cell associations allowing for similar protection from immune surveillance for the central cells as is provided by thrombus formation[21]. Alternatively, these cells may indirectly stimulate platelet aggregation in vivo, which would not be readily detectable in vitro. In general these two studies identified that high platelet aggregating ability is not sufficient for metastasis of non-metastatic cells[41], and indicate that tumor cell-thrombus association is an important determinant in metastatic potential[42].

Interestingly, the splenomegaly identified in tumor-bearing animals was associated with tri-lineage extramedullary hematopoiesis, with a preponderance of megakaryocytic precursors. A similar increase in hematopoiesis was not found in the bone marrow of tumor-bearing mice, which is in accordance with normal murine physiologic response. During stress, the majority of (reactive) hematopoiesis is performed in the murine spleen[27]. Despite the increase in platelet manufacture in the spleen, the number of circulating platelets was significantly decreased in tumor-bearing mice, indicating that the spleen was unable to replenish the platelets being lost.

Several possibilities exist for the cause of the platelet consumption and resulting thrombocytopenia in this case. B16F10 murine melanoma cells express TF on their cell surface[23, 43], therefore the cells within the tumor may activate circulating platelets resulting in thrombus formation at the primary tumor site. B16F10 primary tumors tend be hemorrhagic with a necrotic core void of much secondary structure, and can lead to extensive bleeding with even minor trauma (unpublished observation). In addition, and perhaps most importantly, the presence of a B16F10 primary tumor may stimulate disseminated intravascular coagulation. Disseminated intravascular coagulation in cancer is characterized by activation of the coagulation cascade in the defective tumor vasculature or by pro-coagulant activity on tumor cells, or their membrane components, and is the most common cause of thrombocytopenia in cancer patients[34, 44]. Local fibrinogen and platelet consumption then lead to systemic deficiencies and increased clotting time. Although human patients may be somewhat less susceptible to tumor-associated thrombocytopenia (thrombocytosis is the most common blood abnormality in

cancer patients[34, 35]), mice are known to compensate poorly for blood or platelet loss and easily develop anemia or thrombocytopenia following a minor insult[27]. Interestingly, B16F10 cells have been found to generate large numbers of microvesicles (MV) in vitro; isolation and injection of these MV prior to i.v. injection of B16F10 cells has been linked to increased metastasis[45]. Given the cell surface expression of TF on B16F10 cells,[23] it would be anticipated that these MV would also contain TF. Therefore, it is possible that injection of MV two-hours prior to injection of B16F10 cells[45] could result in an increase in tumor-cell associated thrombus formation, leading to an increase in metastasis. In the presence of an ongoing release of TF-containing MV from a B16F10 primary tumor, it would be anticipated that rather than a pro-metastatic effect of the MV, there would be sustained activation of the coagulation cascade leading to platelet exhaustion and/or depletion, as was seen here. Therefore the release of MV from the B16F10 primary tumor may be partially responsible for the significant reduction in platelet numbers.

The integral role of coagulation in metastasis formation is further indicated by the effect of anti-coagulant therapy in cancer patients. Preliminary clinical trials have shown a significant increase in overall survival of patients randomized to receive LMWH versus placebo[46]. Interestingly, long-term exposure to LMWH in patients with cancer-related thrombosis only increased survival of those patients who did not have metastatic disease at the time of study enrollment[46]. Additionally, large cohort analysis of overall probability of death in those patients diagnosed with cancer as compared to those diagnosed with cancer and thromboembolism, found that approximately 20% of patients with cancer alone and 90% of patients with a combined diagnosis succumbed to their disease within 6 months. It has been indicated that these deaths could be attributed to three possible scenarios: 1) fatal recurrent pulmonary thromboembolism; 2) identification of a coagulation disorder may simply be a surrogate marker for more aggressive malignancy; 3) systemic aberrations in the coagulation pathway may result in a more permissive tumor cell-host interaction giving rise to extensive tumor growth resulting in the early death of the patient[46]. Given these data, further analysis of modulation of the systemic coagulation pathway in cancer patients as a means to prevent metastasis establishment and premature death is warranted.

The development of metastases occurs in the midst of a complex interaction between a primary tumor and the host - the full extent of which remains unknown. There is the potential for a primary tumor to both promote metastatic progression through stimulation of pre-metastatic niche formation, as well as to restrict metastasis through concomitant tumor resistance. In this study, we identified a form of concomitant tumor resistance known as athrepsia, which was in this case due to primary tumor-induced thrombocytopenia and the resulting reduction in tumor-thrombus formation, with inhibition of metastasis. Full understanding of the interplay between a primary tumor, the host and forming metastases will be essential to the development of strategies to inhibit metastatic progression, either before or after the surgical resection of the primary tumor.

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Chapter 4

4 Primary tumor presence restricts metastatic establishment in a murine model of human breast cancer

4.1 Synopsis

Breast cancer is the most common cancer and the second most common cause of cancer-related mortality in Canadian women. These deaths are not due to the presence of a primary tumor however, but are caused by the spread of cancer cells to sites throughout the body. These cells give rise to metastases that are present at the time of diagnosis, or lead to tumor recurrence and patient relapse. Analysis of breast cancer patient data acquired prior to the routine use of adjuvant therapies identified a pattern of breast cancer recurrence that was independent of disease stage at the time of surgery and appears synchronized with the time of tumor resection. It appears that removal of the primary tumor causes a simultaneous initiation of metastatic growth through the release of growth factors during the wound healing process, or that the primary tumor was somehow restricting the growth of metastases and upon tumor resection, metastatic growth is no longer suppressed. Alternatively, formation of a pre-metastatic niche, a cluster of bone marrow host cells that forms a supportive metastatic environment, is initiated by proteins released from a primary tumor. The pre-metastatic niche forms prior to the arrival of metastatic cells and leads to increased tumor cell adhesion and growth. The phenomena of pre-metastatic niche formation and primary tumor suppression of metastasis are contradictory and together they indicate that the interaction between a primary tumor, host systems, and metastatic cells is complex, and remains poorly understood. Previous work in our laboratory has shown that a murine melanoma primary tumor is able to restrict the development of secondary metastasis through alterations in systemic hemostatic factors (Chapter 3). Therefore, we sought to evaluate the effect of an established breast primary tumor on the early development of lung metastases to determine if the metastatic inhibition seen previously also exists in a model of human breast cancer

In this study, we investigated the effect of a breast primary tumor on the early development of lung metastases and found that tumor bearing animals developed significantly fewer lung metastases than tumor-naïve control animals. Tumor-bearing animals showed an increased number of $VEGFR1^{+}$ cells in the lung, potentially indicating BMDC recruitment due to expression of OPN. Preliminary data to determine the mechanism of primary tumor-mediated inhibition of metastasis indicates that successful metastasis may depend on activation of the host hemostatic system which may be impaired in the presence of a primary tumor.

4.2 Introduction

Breast cancer is estimated to account for 5400 deaths in Canada in 2010[1]. These deaths are not due to the primary tumor, but rather because of the spread of tumor cells from the primary tumor to distant sites[2]. Those tumors that have reached a relatively large size and/or have metastasized to several lymph nodes at the time of diagnosis carry a significantly higher risk of recurrence compared early-stage tumors[3].

Interestingly, analysis of patient data accumulated prior to the routine use of adjuvant chemotherapy, has identified a two-peak pattern for recurrence[3, 4]. When all patients are considered, regardless of tumor stage at diagnosis, there is a sharp peak of recurrences at 18 months post-surgery and another broad peak at 60 months[3]. This recurrence pattern was identified in over 12 clinical studies, with varying peak amplitudes, but with an identical timeline[5, 6]. More recent clinical data shows that this first peak no longer exists at 18 months, but rather, the addition of adjuvant chemo- and radiation therapy has shifted the major peak of recurrence to approximately 4 years[7]. The similar patterns of recurrence in all patient groups regardless of disease stage at the time of surgery may indicate a synchronization of metastatic development upon removal of the primary tumor[5]. This synchronization could be due to the release of growth factors during wound healing or a sudden removal of growth suppressive factors, such as angiostatin, with resection of the primary tumor[3, 5].

Concomitant tumor resistance, the ability of a primary tumor to restrict the growth of secondary metastases, has been identified in several pre-clinical models of metastasis and is thought to occur through three potential mechanisms[8]. First, the primary tumor may prime the immune system to assist clearance of metastatic cells. Second, the primary tumor may restrict the growth of distant metastases through production of anti-angiogenic molecules such as angiostatin or TSP[9]. Third the primary tumor may systemically deplete essential host factors, thereby preventing the growth of any other tumors[8]. The first hypothesis would not explain the clinical breast cancer data, as cells shed prior to tumor resection are not cleared by the immune system if they are able to cause recurrence at later time points. The second or third hypothesis could explain the clinical recurrence

pattern however. Removal of the primary tumor would eliminate the growth-suppressive molecule responsible for holding metastases in check[3] or could free up host factors essential for metastatic growth. In both scenarios, removal of the primary tumor would lead to a simultaneous growth initiation of distant metastases due to sudden removal or availability of the key factor(s).

Alternatively, intriguing work published by McAllister, et al.[10] showed that a human breast cancer xenograft did not restrict the growth of other tumors or metastases, but rather was capable of stimulating the growth of an otherwise-indolent transformed mammary epithelial cell line (HMLER-HR). This growth stimulation was due to the activation and mobilization of BMDC by OPN secreted from the stimulating primary tumor. The activated BMDC colonized both the stimulating and indolent tumor sites which allowed for supportive tumor stroma development and increased tumor growth[10]. As further indication that a primary tumor can have a systemic effect, BMDC stimulation by the presence of a primary tumor has also been described during pre-metastatic niche formation. In this case, BMDC mobilization and colonization of distant normal tissues occurs prior to the arrival of metastatic cells, and these BMDC develop into a supportive environment which aids the arrest and development of metastases[11-14].

The pre-clinical identification of pre-metastatic niche formation conflicts with the observation that a primary tumor may hold metastatic growth 'in check'[6, 7]. Together, these phenomena indicate that there is a complex interaction between a primary tumor, secondary metastases and the host environment which remains poorly understood. Previous work in our laboratory has shown that a murine melanoma primary tumor is able to restrict the development of secondary metastasis through alterations in systemic hemostatic factors (Chapter 3). Therefore, we sought to evaluate the effect of an established breast primary tumor on the early development of lung metastases to determine if the metastatic inhibition seen previously also exists in a model of human breast cancer.

Using a variant of the human breast carcinoma MDA-MB-231 cell line with increased metastatic ability (231LN), we found that the presence of a mammary fat pad (mfp)

tumor reduced the number of lung metastases arising from a second intravenous injection. The size of lung metastases that did develop was not affected, indicating that the primary tumor may be affecting initial cell arrest or survival in the lung. Immunohistochemistry analysis of lung tissue found that tumor bearing animals showed increased presence of $VEGFR1^+$ cells, indicating that 231LN cells may be capable of stimulating pre-metastatic niche formation. Despite the presence of the metastasis-supporting $VEGFR1^+$ cells, the number of lung metastases present in tumor-bearing animals was suppressed, therefore the inhibition of metastasis by the primary tumor overwhelms the supportive role of $VEGFR1^+$ cells. Preliminary data to identify the mechanism behind the inhibition of metastasis by a primary tumor indicates that in accordance with previous work from our laboratory, successful metastasis may depend on activation of the host hemostatic system which may be impaired in the presence of a primary tumor.

4.3 Materials and Methods

4.3.1 Cell culture and transfections

MDA-MB-231-luc-D3H2LN (231LN) cells were purchased from Caliper Life Sciences (Hopkinton, MA). They are a variant of the MDA-MB-231 cell line which contain a luciferase-expressing gene and have a propensity to metastasize the lymph nodes, and have a high endogenous OPN production. They were maintained in MEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma, Mississauga, Canada), 1 mM sodium pyruvate (Gibco) and 0.1 mM non-essential amino acids (Gibco) at 37°C with 5% CO_2. Two cell lines were generated from the 231LN cells, one carried no fluorescence marker, the 231LN-scr cells and the other carried a stable red fluorescent tdTomato marker. 231LN-tdTomato cells were generated by nucleofection of the MDA-MB-231-luc-D3H2LN (231LN) with the tdTomato-containing pcDNA3.1 vector with a hygromycin resistance gene, using Amaxa nucleofection technology (Amaxa, Koeln, Germany) according to manufacturer's instructions. Briefly, 1×10^6 231LN cells were resuspended in Cell Line Nucleofector Kit V (Amaxa), mixed with 2 μg cDNA and pulsed with the program X-13, as suggested by the manufacturer. Immediately after nucleofection, cells were transferred into wells containing pre-warmed (37°C) culture

medium (as above). Forty-eight hours post-nucleofection, cells were selected using 750 μg/ml hygromycin (Gibco), to create a stably tdTomato-transfected cell line. All cells were routinely tested and confirmed to be free of mycoplasma contamination using the Mycoplasma Plus PCR primer set (Stratagene, Cedar Creek, TX).

4.3.2 Primary tumor and metastasis assay

Each animal used in this assay received a mfp injection of either 231LN-scr cells or cell vehicle, and all animals received an i.v. injection of 231LN-tdTomato to allow for separation of metastases arising from the primary tumor or i.v. injection. 231LN-scr cells were grown to 70-80% confluence, trypsinized and washed in cold Hanks Balanced Salt Solution (HBSS, Invitrogen). For mfp injection, cells were resuspended in a 50% Matrigel Matrix (BD Biosciences) solution with HBSS at a concentration of 4×10^7 cells/mL and 50 μL (2×10^6 cells) were injected. NIH III (nude-beige, Charles River, Massachusetts) mice were lightly sedated with an intraperitoneal injection of xylazine/ketamine (1.3 mg ketamine and .065 mg xylazine per 20 g body mass) and cells were into the right thoracic mammary fat pad without an incision. As illustrated in Figure 4.1, control animals received a mammary fat pad injection of 50% Matrigel matrix (BD Biosciences) solution with HBSS following the same procedure. Primary tumors were allowed to grow for 28 days, at which point all animals received a second cell injection. Unanesthetized tumor-bearing and tumor-naïve mice received an intravenous (i.v.) injection of 5×10^5 231LN-tdTomato cells in 100 μl HBSS via the lateral tail vein. Animals were sacrificed 6 days following i.v. injection and lung tissue was isolated and snap-frozen to enable quantification of the number of lung surface metastases using fluorescence.

4.3.3 Quantification of lung metastasis

The total number of red-fluorescent metastases was manually determined by a blinded observer on intact lung lobes using an Olympus Inverted IX81 microscope. Area of individual lung metastases was determined using the Olympus FluoView FV1000 coupled to the IX81 motorized inverted system microscope at the Victoria Research Laboratory confocal microscope core facility. Images were acquired with maintained

imaging parameters by a blinded observer and area of each metastasis was determined on fluorescent images using ImageJ[15].

4.3.4 Histology and Immunohistochemistry

Lung tissues were fixed in 10 % neutral buffered formalin and embedded in paraffin. Sections were cut to 4 µm thickness and stained with H&E or with monoclonal rat anti-mouse VEGFR1 (R&D Systems, Minneapolis Mn; #MAB471,) diluted to 10 µg/mL for one hour at room temperature, after heat-induced epitope retrieval. Secondary detection was achieved by linking to biotinylated polyclonal rabbit anti-rat antibody (Dako, Burlington Ontario; #E0468) diluted to 1:500 for thirty five minutes, followed by labeling with StreptABComplex/HRP (Dako, Burlington Ontario; #K0377) and diaminobenzidine (DAB). Positively-stained cells were counted by a blinded, trained observer on an upright microscope on a 40x objective (Olympus BX45).

4.3.5 Western blot analysis of conditioned media

To confirm that OPN secretion from 231LN-scr cells did not differ from the parental 231LN cells, conditioned media was collected, concentrated and used in Western blots as previously described[16]. Briefly, 231LN and 23LN-scr cells were grown to 70-80% confluency, trypsinized and 5 x 10^5 cells were plated on 100 mm dishes. Plates were incubated at 37°C with 5% CO_2 for 24 hours, then media was removed and plates washed twice with PBS. Five millilitres of OPTIMEM was added to each and plates were returned to incubator for a further 24 hours. OPTIMEM was drawn off and concentrated using Amicon Ultra centrifugal filters with a 30 000 dalton molecular weight cut off (Millipore; Billerica, MA). Cells were harvested from plates and counted using a hemocytometer. Using the number of cells, volumes of conditioned media were calculated to represent a specified number of cells (1 x 10^5), allowing conditioned medium from equal numbers of cells to be loaded for Western blotting.

Western blots were carried out using the BioRad Mini Protean II Cell system. Polyacrylamide gels (8%) were loaded with concentrated conditioned media with 5X reducing loading buffer. After electrophoresis, separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham, GE Healthcare; Baie d'Urfe,

QC). Membranes were blocked with 5% milk in TBS-T (tris-buffered saline with 0.1% Tween) for one hour and after rinsing with TBS-T, were incubated with anti-OPN mAb 53 mouse monoclonal antibody (Assay Designs, Enzo Life Sciences; Plymouth, PA) at 1:1000 in TBS-T at 4°C overnight. After rinsing three times with TBS-T, anti-mouse (Amersham) secondary antibody was incubated at room temperature for one hour. After rinsing three times with TBS-T, membranes were incubated with ECL Plus Western Blotting Detection System (Amersham) and exposed to film in a dark room.

4.3.6 Flow cytometry

To compare TF expression between the parental MDA-MB-231 (ATCC), and 231LN-scr and 231LN-tdTomato cell lines, all cells were grown to 70-80% confluency, trypsinized and washed twice with cold flow buffer (PBS + 2% FBS). Cells were resuspended in cold flow buffer (1×10^6 cells/mL) and separated into 100 µl aliquots. Cells were incubated with 3 µl anti-tissue factor antibody (FITC-conjugated anti-human tissue factor, 4508CJ American Diagnostica, Stamford, CT) or 4 µl control antibody to account for non-specific binding (FITC-conjugated Mouse IgG1, 349041 BD Biosciences, San Jose, CA) for 30 minutes with occasional vortexing. Cells were then washed twice with excess volume PBS, resuspended in cold flow buffer prior to analysis on an EPICS XL-MCL flow cytometer (Beckman Coulter, Mississauga, ON). A minimum of 1×10^5 $FITC^+$ events were counted per sample.

4.4 Results

4.4.1 Primary tumor presence decreases lung metastasis

To evaluate the effect of a human breast adenocarcinoma primary tumor on the early development of metastases, we utilized a modified model of experimental metastasis where animals received both a primary and secondary tumor cell injection (Figure 4.1 a). Animals first received a mammary fat pad injection of 231LN-scr cells (primary tumor group) or cell vehicle (tumor-naïve group). After twenty-eight days, when primary tumors had reached ~750 mm^3 (Figure 4.1 b), all animals received a secondary injection of 231LN-tdTomato cells via the lateral tail vein to target cells directly to the lung. The fluorescent label in 231LN-tdTomato cells enabled delineation of metastases arising from

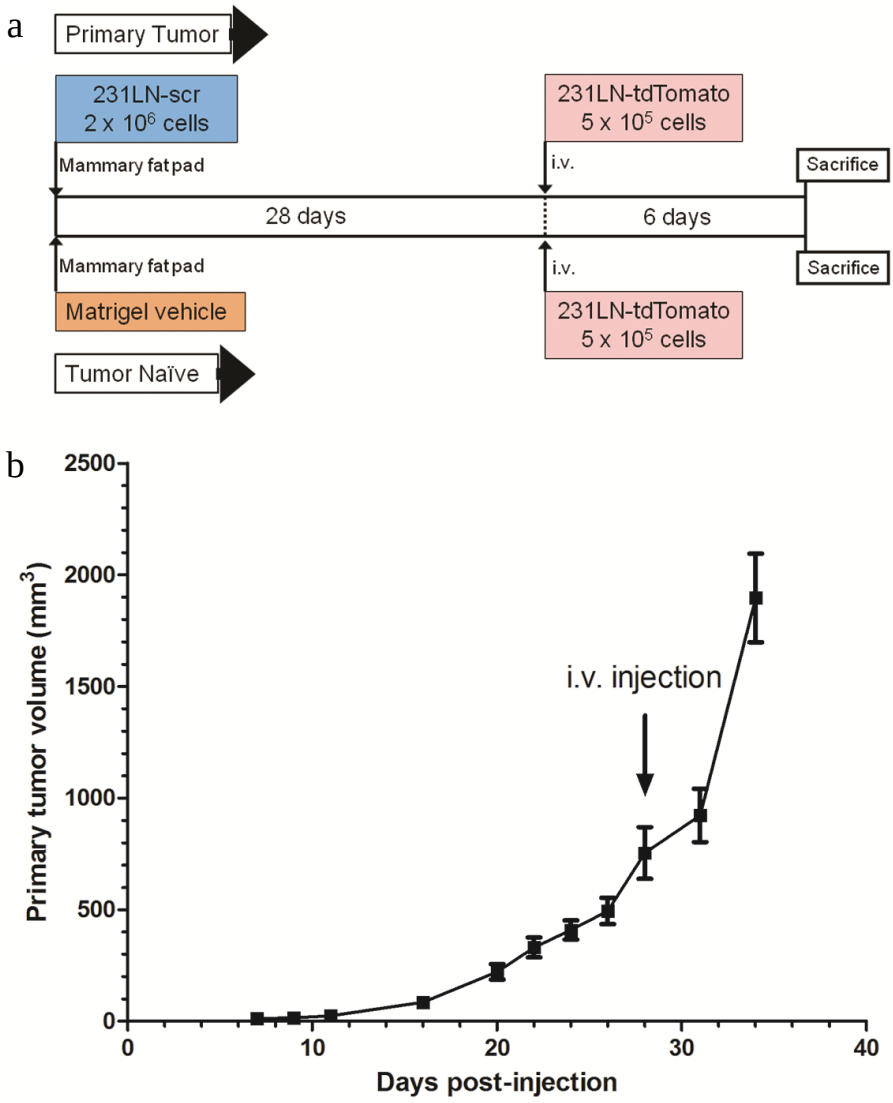

Figure 4.1 Timeline of primary tumor and metastasis injection model.

(a) Experimental model used to analyze the effect of a primary tumor on the early development of metastases. Animals first received a mammary fat pad injection of Matrigel (cell vehicle) or 231LN-scr cells suspended in Matrigel. Following primary tumor establishment all animals received an i.v. injection of 231LN-tdTomato cells. Six-days following i.v. injections all animals were sacrificed and lung and primary tumor tissues were collected. (b) Primary tumor growth of 231LN-scr cells. Animals received an i.v. injection of 231LN-tdTomato cells on day 28 of tumor growth when primary tumors were approximately 750 mm^3.

the i.v. injection. We found that the presence of a primary tumor significantly inhibited experimental lung metastasis, as six days following secondary injection there were fewer red-fluorescent lung surface metastases in primary tumor-bearing mice as compared to tumor-naïve animals (Figure 4.2 a). There was no difference in the size of those lung metastases that did form in the primary tumor-bearing and tumor-naïve group (Figure 4.2 b). Histological analysis of lung tissue identified single tumor cells and small clusters of tumor cells in both tumor-naïve and tumor-bearing animals (Figure 4.2 c and d), in agreement with the small size of metastases determined using fluorescence microscopy.

4.4.2 BMDC recruitment to the lung

Previous reports have shown that expression of OPN from a primary tumor can stimulate mobilization of BMDC to colonize primary tumor stroma[10]. It has also been shown that proteins secreted from a primary tumor can stimulate VEGFR1$^+$ BMDC recruitment to form the pre-metastatic niche, which may lead to increased metastatic cell arrest and growth[12, 17]. Therefore, we performed immunohistochemistry to determine if VEGFR1$^+$ cells were present in the lung of primary tumor-bearing or tumor-naïve animals and if these cells were found in association with 231LN-tdTomato cells. Quantification of immunohistochemistry slides found significantly more VEGFR1$^+$ cells in lung tissue of primary tumor-bearing animals as compared to tumor-naïve controls (Figure 4.3 a). Additionally, large clusters of more than fifteen VEGFR1$^+$ cells were found in primary tumor-bearing animals, potentially indicating the presence of a pre-metastatic niche (Figure 4.3 b). These large clusters were not visualized in control lung tissue. Tumor cells were found associated within the clusters of VEGFR1$^+$ cells, either in small groups of breast cancer cells or as single tumor cells in both primary tumor-bearing (Figure 4.3 c) and tumor-naïve animals (Figure 4.3 d).

4.4.3 OPN expression by 231LNscr cells

To determine if the increased presence of VEFGR1$^+$ cells in the lung was due to secretion of the BMDC-stimulating protein, OPN, Western blot analysis of conditioned media samples was performed. It was found that 231LN-scr cells maintained a similarly-high level of OPN secretion as the 231LN cells after extended in vitro culture (Figure 4.4),

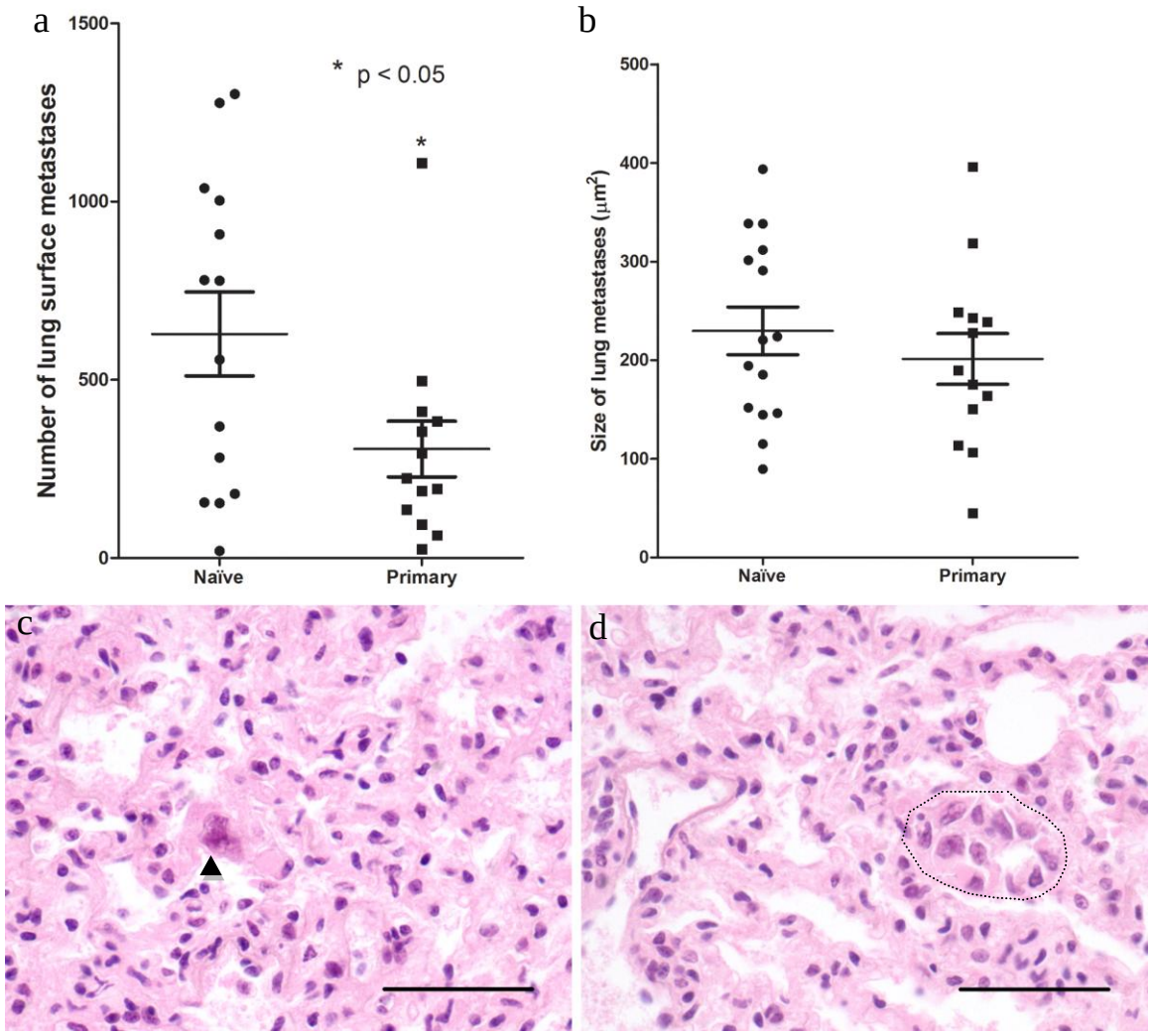

Figure 4.2 Primary tumor presence significantly reduced pulmonary metastasis

(a) The presence of a 231LN-scr primary tumor significantly reduced the number of 231LN-tdTomato lung metastases as compared to tumor-naïve controls (n = 13-15/group; t-test, $p < 0.05$). (b) Lung metastases in primary tumor-bearing animals were equivalent in size to their tumor-naïve controls (n = 13-15/group; t-test, $p > 0.05$). (c) Histological analysis showed isolated tumor cells (arrowhead) or (d) small clusters of tumor cells (outline) in both primary tumor-bearing and tumor-naïve animals. Scale 50 μm.

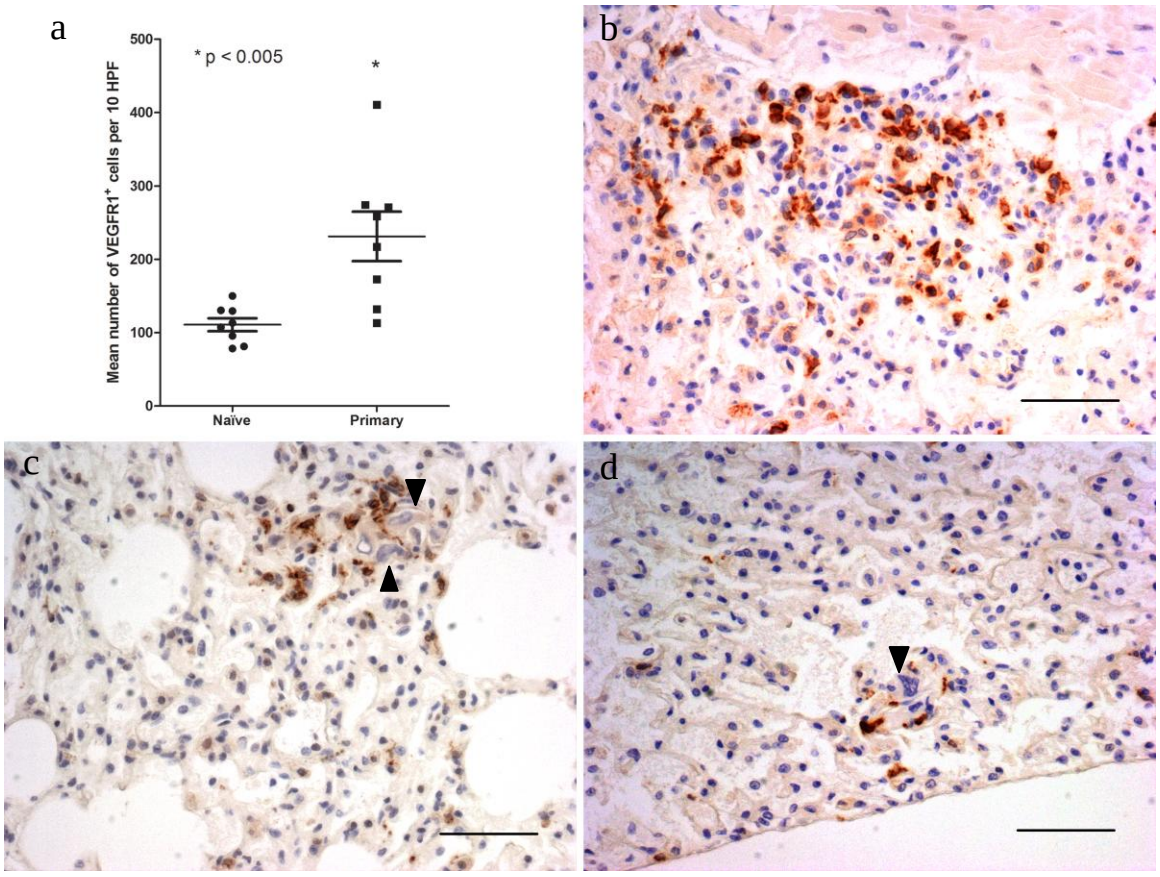

Figure 4.3 Primary tumor presence increases the number of VEGFR1$^+$ cells in the lung.

(a) Quantification of immunohistochemistry staining for VEGFR1 identified a significant increase in the number of VEGFR1$^+$ cells in the lung of primary tumor-bearing animals (n = 8/group, t-test $p < 0.005$). Large clusters of more than 15 VEGFR1$^+$ cells were only visualized in primary tumor-bearing mice (b) and tumor cells (arrow heads) were found associated within the clusters of VEGFR1$^+$ cells in both primary tumor-bearing (c) and tumor-naïve animals (d).

therefore the increased presence of VEGFR1$^+$ cells may be due to BMDC recruitment stimulated by OPN secretion. Interestingly, despite the increased presence of potentially metastasis-promoting VEGFR1$^+$ cells[17], primary tumor-bearing animals showed reduced metastasis (Figure 4.1 a).

4.4.4 Tissue Factor expression

Previous work in our laboratory has shown that a B16F10 murine melanoma primary tumor inhibits metastatic establishment through depletion of circulating platelet numbers (Chapter 3). High levels of TF on the surface of B16F10 cells[18] results in thrombus formation following arrest of these cells in the lung, which is essential for metastatic spread (Chapter 3). Therefore, expression of TF on 231LN-scr and 231LN-

tdTomato cells was evaluated by flow cytometry. It was found that in accordance with previously published MDA-MB-231 parental cell data[19], MDA-MB-231 cells (Figure 4.5 a) as well as both the 231LN-scr (Figure 4.5 b) and 231LN-tdTomato (Figure 4.5 c) cell lines indeed express TF, indicating that these cells should be capable of stimulating thrombus formation, and successful thrombus formation may play a key role in the establishment of lung metastases, as has been found previously for MDA-MB-231[19].

4.5 Discussion

The ability of a primary tumor to impact the development of metastases can take two forms. Through stimulation of a pre-metastatic niche a tumor can increase metastatic establishment, or through concomitant tumor resistance a tumor can prevent successful metastasis. In this study, we determined that the presence of a 231LN-scr mammary fat pad primary tumor significantly inhibited the early establishment of 231LN-tdTomato lung metastases. This inhibition is likely due to a decrease in sustained cell arrest or survival in the lung vasculature rather than an inhibition of growth, as the size of those metastases that did develop was unaltered by the presence of a primary tumor. As has been demonstrated with B16 cells[17], the 231LN-scr primary tumor led to a significant increase in the number of VEGFR1$^+$ cells in the lung, potentially due to OPN secretion and stimulation of BMDC recruitment. The presence of VEGFR1$^+$ cells has previously

been reported to increase lung metastasis through initiation of pre-metastatic niche formation, yet in this study, the presence of a primary tumor inhibited metastasis. The mechanism behind the significant decrease in metastasis remains unclear, but appears to override the metastasis-stimulating effect of $VEGFR1^+$ cells.

As outlined in Chapter 3, the presence of a B16F10 melanoma primary tumor was found to inhibit lung metastasis formation due to a significant reduction in the circulating platelet numbers in tumor-bearing animals. This led to an impairment of tumor cell surface-thrombus formation following i.v. injection of B16F10-LacZ cells. Restoration of the platelet number re-established thrombus formation as well as lung metastasis. Given that the B16F10[18], 231LN-scr, and 231LN-tdTomato cells all express TF on the cell surface and the importance of thrombus formation in successful B16F10 metastasis, it is possible that thrombus formation may play a key role in metastasis of 231LN cells. The presence of a 231LN-scr primary tumor may result in a similar depletion of circulating platelets as seen in the B16F10 model. Analysis of circulating platelet numbers in 231LN-scr tumor bearing animals is ongoing in our laboratory.

The inability to form a thrombus during metastasis in the B16F10 model system may lead to an increase in NK-mediated cell killing[20] as C57Bl/6 mice are fully immunocompetent. In the 231LN model however, the NIH III animals do not have functional T-cells, B-cells or NK cells[21], making it improbable that increased immune surveillance in the absence of thrombus formation leads to the reduction in metastasis seen here. Thrombus formation is also known to result in increased stable cell adhesion in the lung[22, 23], therefore the lack of thrombus formation may result in an increased number of cells passing through the lung capillary bed or increased cell death due to anoikis from lack of stable adhesion[24]. Unlike other examples of concomitant tumor resistance where the release of anti-angiogenic molecules restricts metastatic growth[9], it is not anticipated that inhibition of angiogenesis is responsible for the decrease in metastasis seen here. First, there was no size difference between metastases quantified in the tumor-bearing and tumor-naïve animals. Second, metastases found in both primary tumor-bearing and tumor-naïve animals were very early in development and were less than 1 mm in diameter and therefore have not reached a size where they need to recruit their own blood supply[25, 26].

Additionally, previous work in a fibroscarcoma model has found a biphasic pattern of concomitant tumor resistance[27]. It was found that an immunogenic tumor (MC-C fibrosarcoma) is capable of stimulating increased immune surveillance of metastatic cells when the primary tumor is less than 500 mm^3 and is capable of restricting metastatic growth through inhibition of angiogenesis when the same tumor was greater than 2000 mm^3 [27]. Primary tumors between 500 and 1500 mm^3 had no effect on metastatic progression[27]. In the 231LN model used here, primary tumors were approximately 750 mm^3 at the time of the second injection and therefore were within the range when no significant effect on metastasis was identified previously. It is unknown if the MC-C fibrosarcoma cell line expresses TF or if it is highly thrombogenic, though a difference in TF expression may account for the differential pattern of inhibition seen between the MC-C and the B16F10 (Chapter 3) and the 231LN-scr cell lines.

The MDA-MB-231 cell line has previously been shown to stimulate BMDC migration to tumor sites[10], and to the pre-metastatic niche[12]. Expression of OPN from the MDA-MB-231 cell line was found to mobilize BMDCs and stimulated their colonization of primary tumor sites, which led to growth instigation in an otherwise-indolent cell line[10]. Additionally, expression of lysyl oxidase by a MDA-MB-231 primary tumor led to increased collagen IV crosslinking and increased $CD11b^+$ myeloid cell recruitment to the lung[12]. These essential components of the pre-metastatic niche increased metastatic cell recruitment and tumor cell invasion[12]. Here, we found a significant increase in $VEGFR1^+$ cells in the lung of primary tumor-bearing animals, but this increase was unable to promote metastasis, or was unable to overcome the inhibitory influence of the primary tumor.

Histological analysis identified the presence of $VEGFR1^+$ cells in both tumor-naïve and primary tumor-bearing animals, and association of these cells with tumor cells in both animal groups. Larger numbers of $VEGFR1^+$ cells and the presence of clusters of more than fifteen $VEGFR1^+$ cells were only identified in primary tumor-bearing animals, potentially indicating the presence of a pre-metastatic niche. This niche formation could be due to expression of OPN from the 231LN-scr primary tumor. Further analysis of

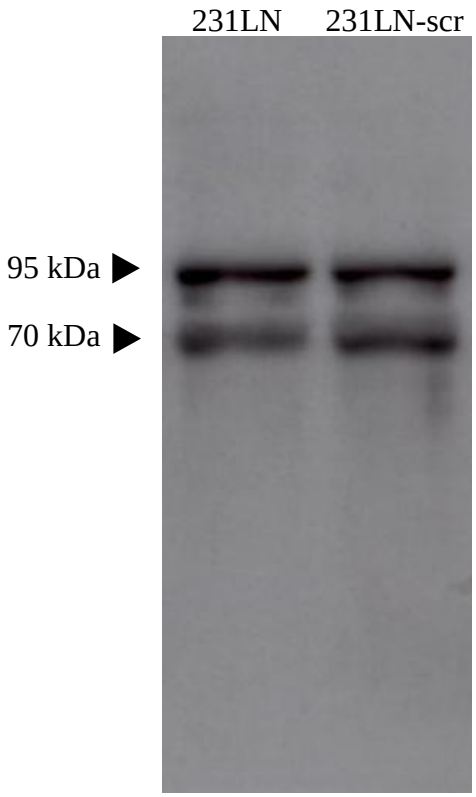

Figure 4.4 Western blot analysis of conditioned media from 231LN and 231LN-scr cells.

23lLN and 231LN-scr cells were cultured in vitro for 4-weeks prior to isolation of conditioned media to determine secreted OPN levels. Control transfection with a scrambled shRNA sequence does not appear to have affected OPN expression, as similar levels of OPN secretion were found in the 231LN and 231-scr cell conditioned media. Multiple bands of OPN protein are present between 70 and 95 kDa.

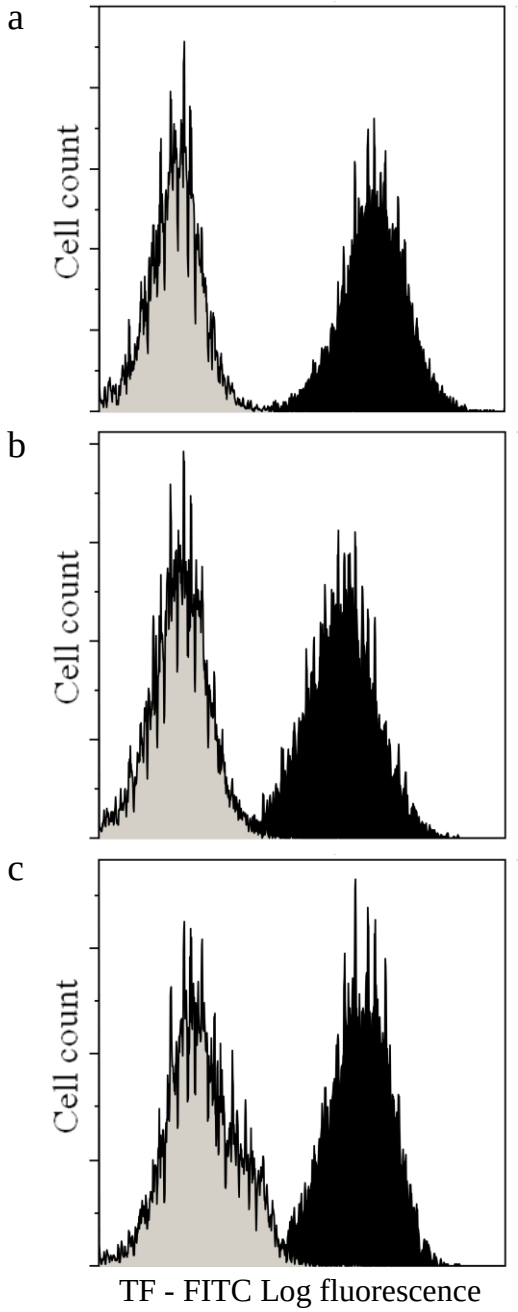

Figure 4.5 Flow cytometry analysis of TF expression.

Analysis of cell surface expression of TF by flow cytometry showed that all MDA-MB-231 (a), 231LN-scr (b) and 231LN-tdTomato (c) cells express high levels of TF. Grey curves represent control IgG1 antibody; black curves represent TF-specific antibody.

lung tissue is required to determine if other hallmarks of the pre-metastatic niche are present, such as increased FN deposition from fibroblasts, presence of $VEGFR2^+$ or the presence of $CD11b^+$ myeloid cells. Additionally, we are analyzing the 231LN-scr cell line to determine the expression level of several molecules thought to be important in stimulating pre-metastatic niche formation. Along with OPN, TNFα, TGFβ, VEGF and PlGF have all been implicated in BMDC activation and mobilization resulting in pre-metastatic niche establishment[14].

Along with the metastasis-promoting protein, OPN, 231LN cells were shown to express TF on their cell surface, indicating that they may depend on thrombus formation for successful metastasis. Establishing if the presence of a 231LN-scr primary tumor reduces circulating platelet numbers, as seen in the B16F10 model (Chapter 3), will be essential to determine if a common mechanism of metastatic inhibition exists in those tumor types that express high levels of TF. If so, this would indicate that initiation of anti-coagulant therapy may prevent metastatic establishment prior to primary tumor resection, and extended treatment with low dose anti-coagulant therapies may provide long-term clinical benefit.

The mechanism behind the decrease in metastasis identified here remains unclear and is being investigated in ongoing work in our laboratory. Other ongoing work to elucidate the mechanism behind metastatic inhibition includes time course analysis following metastatic cell injection to determine if the presence of a primary tumor inhibits initial cell arrest, sustained cell arrest, or tumor cell survival in the lung. Extra-pulmonary organs will also be evaluated to determine if iv-injected 231LN-tdTomato cells do indeed pass right through the lung capillary bed and reach distant viscera more frequently in primary tumor-bearing animals. Previous work with anticoagulants has shown that inhibition of coagulation can lead to impaired cell arrest and reduced pulmonary metastasis[28].

Understanding the mechanism behind primary tumor inhibition of metastasis will prove invaluable in elucidating the interactions between primary tumor, the host, and metastases. Significant breakthroughs in treatment options could result, as it may be

possible to restrict metastatic growth by providing or removing the essential factor(s) following primary tumor resection, leading to continued metastatic inhibition.

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Chapter 5

5 General Discussion

5.1 Thesis summary

The interaction between tumor cells and the host is essential at all stages of cancer progression, but is exceptionally important during metastasis. As cells move into the host vasculature and are carried to distant organs, they encounter all new environments. To withstand the challenges that these environments present, tumor cells must possess a means of exploiting or avoiding the host response. This body of work demonstrates that metastatic progression depends not only on the innate characteristics of the tumor cells, but also on their ability to manipulate host systems. Intriguingly, the relationship between host and tumor is not a simple tumor (promotion) versus host (inhibition), as this work also demonstrates that in some cases it is the host which can promote metastasis, while the primary tumor may inhibit metastatic progression. A major component of the tumor-host interaction is the host hemostatic system, which plays a key role in metastatic progression.

It has been well established that many cell lines (LLC and B16 in particular) depend on thrombus formation for successful metastasis[1-5]. Direct activation of circulating hemostatic factors through expression of TF by tumor cells is a key determinant of metastatic potential by inhibiting NK cell-mediated clearance of micrometastases[4, 5]. Indeed, inhibition of thrombus formation through treatment with anti-coagulants can lead to a significant inhibition of pulmonary metastasis[6, 7]. In accordance with the important role of hemostatic factors on metastasis, in Chapter 2 we demonstrated that treatment with the anti-fibrionolytic agents aprotinin and EACA stabilized the interaction between thrombi and tumor cells in the lung vasculature and increased pulmonary metastasis of TF-expressing B16F10 cells. Importantly, previous pre-clinical analysis of aprotinin has shown varying impacts on metastasis, depending on the model and cell line used, without giving insight into its mechanism of action[8-12]. Several studies found that aprotinin could decrease metastasis potentially through inhibition of tumor cell extravasation[8, 12] but the

work presented in Chapter 2 definitively shows that the survival benefit afforded by increased interaction with thrombi while in the host vasculature outweighs any anti-metastatic effects.

Chapter 2 also describes the development of a novel and highly-useful fluorescence imaging technique where the interaction between tumor cells and thrombi are visualized in the intact mouse lung. Through maintenance of experimental and imaging parameters, the confocal images acquired can be used for quantification of this interaction. This straightforward imaging protocol was also utilized in Chapter 3 to investigate the impact of a primary tumor on the association between thrombi and metastatic cells and is easily modified to apply to other model systems.

The data presented in Chapter 2 firmly establishes the importance of hemostasis in tumor cell-host interactions during metastasis. In the clinical setting however, the interaction between host and tumor cells may also be modulated by the presence of a primary tumor. Pre-clinical investigation into the effect of a primary tumor on metastasis has shown that the fate of metastatic cells can be largely dependent on the presence of a primary tumor[13, 14]. Through stimulation of BMDC to form a pre-metastatic niche a primary tumor may promote metastasis[15], or it can cause a significant reduction in metastatic progression through a phenomenon known as concomitant tumor resistance[13]. Differential expression of metastasis-promoting (OPN[16], lysyl oxidase[17]) or -inhibiting factors (angiostatin, TSP[18]) from the primary tumor have been suggested to be responsible for these disparate effects. In Chapter 3, we present a previously under-studied mechanism behind concomitant tumor resistance, athrepsia – where the primary tumor usurps an essential host resource thereby preventing metastatic growth[19]. In this study, we found a B16F10 primary tumor-induced thrombocytopenia in the host, which led to insufficient metastatic tumor cell-thrombus association. Given the substantial benefit of this interaction to metastasis, as identified in Chapter 2, it was not surprising that inhibition of this thrombus association with metastatic cells by the primary tumor resulted in a reduction of lung metastasis. Importantly, re-establishment of a normal circulating platelet number was able to restore lung metastasis levels to that seen in tumor-naïve animals.

Given that athrepsia has not been well established as a mechanism for concomitant tumor resistance, there was concern that the phenomenon observed in Chapter 3 was specific to the B16F10 cell line. Therefore, in Chapter 4 we sought to determine if a similar phenomenon existed in a murine model of human breast cancer.

Utilizing a variant of the MDA-MB-231 human breast cancer cell line (231LN) we evaluated the effect of a primary tumor on the development of metastases. Intriguingly, the same pattern of metastatic inhibition by a primary tumor was identified in the 231LN model. Additionally, the 231LN cells were found to express high levels of TF, indicating that thrombus association may play a key role in their ability to form successful metastases. An important difference between the B16F10 and 231LN models is the immunocompetency of the host. B16F10 cells are syngeneic to and grow well in the fully immunocompetent C57Bl/6 mouse strain. The 231LN cells, however are grown in the NIH III strain, which have impaired T-, B- and NK cell function[20]. Although the essential role for tumor cell-associated thrombi in metastasis in the B16F10 (syngeneic) model may be related to increased NK cell surveillance of those cells not protected by thrombi[5], in the 231LN (NIH III) model this same tumor cell surveillance would not be expected, given the lack of NK cell function. It is possible that in the 231LN model, a decrease in thrombus formation leads to a reduction in metastatic cell retention in the pulmonary vasculature[7]; ongoing investigation of early time points following i.v. tumor cell injection will evaluate this possibility.

Interestingly, the presence of either a B16F10 or 231LN primary tumor led to the development of fewer lung metastases, yet only in the B16F10 model were those lung metastases of a smaller size. In both models, animals were sacrificed six days following i.v. injection, yet the mean B16F10-LacZ metastasis size was approximately 18000 μm^3, whereas the 231LN-tdTomato metastases were only 300 μm^3. The B16F10 cell line is exceptionally aggressive, and most metastases had begun to form small colonies at the time of sacrifice. The 231LN-tdTomato cells however, existed mainly as groups of 3-4 cells without showing signs of colony formation or extensive growth. Therefore, it is possible that the 231LN primary tumor would also inhibit the growth of lung metastases,

but this effect was not yet visualized because of the delayed growth initiation as compared to the B16F10 cells.

Identification of concomitant tumor resistance in the both the B16F10 and 231LN models was somewhat surprising, given the work showing that primary tumors arising from both the B16[15] and MDA-MB-231[16, 17] cell lines are capable of stimulating metastasis through BMDC mobilization. Intriguingly, we identified an increase in VEGFR1$^+$ cells in the lung of 231LN primary tumor bearing animals. It is not immediately clear why in our study a 231LN primary tumor leads to an inhibition of metastasis, given the similar effect on VEGFR1$^+$ cells as in the work of Kaplan et al.[15] and Erler et al. [17], but it may be due to differences that arose following the in vivo passage and transfection of the 231LN cells or due to inherent differences between mouse strains used. Our work was carried out in NIH III animals with impaired T-, B- and NK-cell function[20] whereas work published by Erler, JT et al.[17, 21] was carried out in nude (nu/nu) animals lacking T-cells[20]. Importantly, it has been shown that the MDA-MB-231 cells[22], 231LN and 231LN-scr cells all express OPN, a key secreted protein required for BMDC mobilization and colonization of distant tissues. It is possible that the pre-metastatic niche formed in response to 231LN-scr cells does not contain other essential components such as FN or CD11b+ myeloid cells[15, 17], therefore as part of ongoing work within our laboratory we are analyzing the lung tissues of primary tumor-bearing and tumor-naïve animals to potentially identify other facets of the pre-metastatic niche.

The work presented in this thesis illustrates the importance of tumor cell-host interactions in the establishment of metastases. The roles of each player are not clearly defined as tumor versus host with the tumor stimulating metastatic progression and the host striving to inhibit it – as shown in Chapters 3 and 4, the primary tumor may actually inhibit metastasis formation through alterations in host hemostasis. Metastatic progression appears to exist in a delicate balance between promotion and inhibition of metastasis by the primary tumor through interaction with host systems. Further understanding of this ability of a primary tumor to manipulate hemostasis will be invaluable in gaining insight into tumor progression and the global nature of metastasis.

5.2 Experimental Implications

5.2.1 Hemostasis in cancer progression

Most cancer patients with metastatic disease also show some degree of coagulopathy[23, 24]. Perturbations in hemostatsis may occur as a result of surgery, reduced patient mobility, chemotherapeutics or anti-angiogenic drugs, or because of blood flow disruptions through the tumor(s) due to aberrant vascular structures[25]. Additionally, as tumors become increasingly aggressive, there can be a pro-coagulant conversion of cancer cells characterized by increased expression of TF or CP. Several oncogenes are responsible for driving transcription of TF, and for increasing microvesicle release, which can also drive systemic coagulopathy (reviewed in [25]). Investigation of the role of TF in cancer progression has shown that TF expression can be a marker of tumor stage and severity, as high TF expression correlates with poor patient outcome in colorectal and prostate cancer. Additionally, it can be used as a predictor of pro-thrombotic risk[26]. Pre-clinical investigation has shown that TF expression is not a key determinant of primary tumor-forming ability, but can be essential for pulmonary metastasis[4, 25]. In Chapter 3 we identify an additional effect of TF-expressing cancer cells – that pro-coagulant activity of a primary tumor can perturb host hemostasis leading to thrombocytopenia, prior to the involvement of metastatic cells. Given the striking effect of thrombocytopenia on the association between tumor cells and thrombi, and the subsequent inhibition of metastasis seen in Chapter 3, clinical treatment to mimic this effect with anti-coagulants or through platelet depletion could lead to a similar inhibition of metastasis. Identification of those patients with high TF expression in primary tumor tissue or on circulating microvesicles might identify those patients who would benefit from anti-coagulant therapy, even in the absence of symptoms of altered hemostasis. Early treatment of with anti-coagulants might prevent metastasis from a primary tumor that has acquired a pro-thrombotic phenotype but has not yet metastasized. It may also prevent the formation of VTE deep vein thromboses by identifying patients who would be prone to thrombotic events prior to symptom presentation.

Additionally, knowledge of TF expression prior to tumor resection may allow the physicians to make an informed decision regarding the inclusion of anti-fibrinolytic

agents at the time of surgery. If a patient is identified as having a pro-thrombotic expression pattern, it is more likely that those cells shed from that primary tumor will exploit host hemostatic systems to successfully metastasize. By the same reasoning, it may be also be advisable to avoid treatment with agents that would stabilize the interaction between metastatic cells and thrombi, as shown in Chapter 2, as stabilization of this interaction may lead to a significant increase in metastasis.

5.2.2 Concomitant tumor resistance and murine models of metastasis

Investigation of metastasis is generally performed using two types of experimental models, each with their benefits and challenges[27]. In a spontaneous metastasis model, a primary tumor is genetically induced or injected into an orthotopic or subcutaneous site and the animal is observed over time to identify signs of metastatic disease. The spontaneous metastasis model is the most clinically relevant of current models used, as it most closely mimics patient disease, with primary tumor development followed by metastatic progression. The major drawback, however is establishing a timeline of metastasis formation so that tissues can be analyzed at the best stage of metastasis development. In well-established models, the timing of metastatic progression is known and animals can be sacrificed at essential time-points, but establishing this information takes a great deal of time and a large number of animals. Additionally, spontaneous metastasis models generally take an extensive length of time to allow for primary tumor development and then metastatic progression. Finally, the inability to measure the number of cells that have been shed to distant organs makes the spontaneous metastasis model difficult to quantify. To address these shortcomings, experimental metastasis models were developed. In these models, cells are introduced as a suspension directly into the circulation, with the site of injection varying by the organ of interest. To investigate lung metastasis cells are injected via the lateral tail vein. To target the liver, cells are injected via the mesenteric vein and for a global analysis of metastatic outcome, cells are injected via the left ventricle of the heart. Experimental metastasis models mimic the shed of cells into the vasculature and allow for direct quantification of the number of cells that survive and begin to grow[27]. Additionally, co-injection of a known ratio of inert micron-sized beads (microspheres) enables comparison between time-points

to determine the proportion of injected cells that progress from single cells to micrometastases to macrometastases[28, 29]. The major drawback of these models is that they inject a large bolus of cells at a single time, rather than truly mimicking a steady release of cells into the vasculature as is seen clinically. They also bypass the earliest stages of metastasis where cells leave the primary tumor and enter the vasculature. Additionally, the potential impact that a primary tumor may have on metastatic progression is unable to be investigated.

The data presented in Chapter 3 and 4 indicates that pre-clinical investigation of metastasis without the presence of a primary tumor does not allow for analysis of all potential players involved. Evaluation of metastasis using both spontaneous and experimental metastasis models is essential, but may not be the most relevant, especially when testing new therapeutic agents. It is possible that the effect of the agent on metastasis would be tempered by the presence of a primary tumor. Yet relying on a spontaneous model for this type of investigation would be further complicated by the effect that the agent was having on the primary tumor itself. Analysis of a new agent in a spontaneous metastasis model would potentially lead to a reduction in metastasis, but this reduction would be less easily understood due to potential reduction in primary tumor size. Additionally, the step in metastasis affected by the therapeutic would be difficult to identify. Therefore, the use of a model that provides both the primary tumor and the ability to quantify and analyze metastatic outcome through a bolus injection of a known number of cancer cells may provide information not available when utilizing a spontaneous or experimental model alone.

This thesis identifies the ability of a primary tumor to restrict metastatic growth in two well-studied cell lines. Interestingly, the mechanism behind the identified concomitant tumor resistance does not align with the more commonly reported mechanisms, rather it provides insight into the extensive role that the host hemostatic system plays in metastasis. Also, previous studies have shown that both the B16[15] and MDA-MB-231[17] cell lines can instead stimulate metastasis progression through release of specific factors that cause BMDC mobilization to the primary tumor site or to the pre-metastatic niche. Identification of an increase in $VEGFR1^{+}$ cells in the lung of 231LN-scr primary tumor-

bearing animals indicates that it is possible that the 231LN (and perhaps also the B16F10) primary tumors are causing BMDC mobilization, but that the significant role of hemostasis in metastasis formation is able to over-ride any benefit provided by the recruited BMDC.

5.2.3 Clinical implications

The work presented in this thesis emphasizes that changes in patient homeostasis are not just an unfortunate complication of malignancy. Rather, clinical identification of VTE or other thrombotic events indicates that the host hemostatic system is being engaged by a primary tumor or metastatic cells. These changes lead to the morbidity and mortality associated with thrombotic events, but also to alterations in tumor progression. It is also important to recognize that due to the holistic nature of metastatic disease, treatment of one facet of patient health can have an impact on overall patient outcome, potentially beyond the expected mechanism of the treatment. As shown in Chapter 2, treatment of cancer patients undergoing surgery to remove a primary tumor with an anti-fibrinolytic agent will likely reduce the risk of blood loss and transfusion requirements during surgery[30], but may also lead to increased metastasis of those cells shed from the primary tumor. Additionally, development of chemotherapy-induced thrombocytopenia is treated quickly to restore normal platelet numbers[31]. The work presented in Chapter 3 would suggest that maintaining low platelet numbers could significantly reduce metastatic progression. Additionally, chemotherapeutic efficacy has been found to be increased in thrombocytopenic animals due to increases in vascular permeability allowing for increased drug delivery[32]. Induction of thrombocytopenia led to increased tumor hemorrhage, but no other blood loss, and allowed for a 40% increase in drug localization in the tumor. Therefore, though thrombocytopenia represents a significant complication in patient treatment, it may also provide an opportunity to increase therapeutic efficacy.

In evaluating overall status of disease in a cancer patient, platelets represent a particularly valuable resource. Platelets are known to actively sequester VEGF, platelet factor 4 and TSP released from tumor cells[33]. It has been noted that despite normal levels in the plasma or serum of the patient, platelet levels of these factors are already elevated, even when tumors are < 1 mm[34]. Therefore, analysis of platelet granules to identify markers of

aggressive disease could provide advance notice of pending disease progression. Additionally, several clinical studies have identified an improvement in cancer patient outcome following long term treatment with anti-coagulants, especially for those patients without metastases at time of entry to the clinical trial[35].

This thesis suggests that identification and treatment of malignancy-associated complications should be investigated beyond the symptom alone. Each change in overall patient well-being could represent a progression in disease state. Overall, this thesis illustrates that metastasis is a complex process that is affected by a balance of interplay between tumor and host factors. Investigation of individual proteins on tumor cell behavior in vitro, while necessary, is not sufficient to identify the true mechanisms behind disease progression. Many competing influences exist in vivo, therefore investigation of cancer progression in model systems that most closely mimic the clinical situation is essential. Through understanding of the effect of cancer on the host, novel treatments that prevent the exploitation of the host by tumor cells or bolster host-mediated tumor cell killing could be developed.

5.3 Future directions

The focus of cancer metastasis research has previously been on the innate properties of tumor cells which allow for successful metastasis. As described in this thesis, the interaction between a primary tumor, the host and metastatic cells is extensive and can be a major determinant of disease progression. The innate characteristics of a tumor cell that lead to successful metastasis may in fact be those that provide the cell the ability to exploit host systems and tissues. Through identification of the major components of the host which are involved in metastasis, such as the hemostatic system, novel characteristics and important attributes of aggressive tumor cells will be identified, and could provide novel treatment avenues.

The concept of concomitant tumor resistance has been recognized for decades[13], yet has not been fully evaluated to determine the potential mechanisms behind the extensive metastatic inhibition identified. Importantly, concomitant tumor resistance does not represent simply a pre-clinical phenomenon, as it has been found in cancer patients that

resection of a primary tumor can lead to an explosive outgrowth of previously undetected metastases (reviewed in [36]). As discussed in Chapter 4, the timing of breast cancer recurrence may indicate a synchronization of tumor cell growth that aligns with tumor resection[37]. Through investigation of concomitant tumor resistance, understanding of the process of angiogenesis was fundamentally altered by identification of angiogeneisis inhibitors[38]. Unfortunately, these inhibitors did not show great clinical success, yet the understanding gained has revolutionized understanding of normal and tumor angiogenesis and metastasis.

In this thesis, I identified that two different tumor cell lines are capable of inducing concomitant tumor resistance. It is known that the presence of cancer can alter the host hemostatic system but it has not been previously recognized that a primary tumor could restrict metastatic growth through manipulation of hemostasis. Therefore the mechanism behind this inhibition of metastasis does not align with previously elucidated mechanisms of concomitant tumor resistance involving immune-mediated rejection of metastatic cells or growth suppression through anti-angiogenic factors released from the primary tumor. The B16F10 cell line is non-immunogenic, and the 231LN cells are grown in immunocompromised mice, therefore exposure to the primary tumor would not be expected to increase surveillance of secondarily injected cells. Additionally, in both the B16F10 and 231LN model, animals were sacrificed at an early time-point following the second injection. Developing metastases remained very small (18000 μm^3 and 300 μm^3 respectively) and were well below the 1 mm size where a tumor must recruit its own vasculature[39, 40]. Therefore, anti-angiogenic factors released from the primary tumor would not yet have had an effect on metastatic establishment. Changing perspective and considering malignancy as a holistic disease will aid current understanding of the tumor impact on the host, and could lead to different treatment avenues. Thorough investigation of the impact of a primary tumor on metastasis of secondarily injected cells will provide great insight into potential new means to restrict metastatic growth. It is essential to understand the relationship between concomitant tumor resistance and pre-metastatic niche formation. It is likely that differential expression of key factors will lead to either metastatic inhibition or promotion; it is essential that these factors be identified and investigated to determine their role in clinical cancer progression. Interestingly, OPN

expression has been linked with BMDC mobilization[16], and the MDA-MB-231 cell line has been found to stimulate pre-metastatic niche formation, leading to increased metastasis[17]. In this thesis we show that using a variant of the MDA-MB-231 line (231LN), there is an increased number of VEGFR1$^+$ cells present in the lungs of primary tumor-bearing animals, and induced larger clusters of these cells than in tumor-naïve animals. These clusters were often associated with tumor cells and may indicate the presence of a pre-metastatic niche, yet no overall increase in metastasis was identified. It appears that the metastatic inhibition induced by the primary tumor is able to overcome the promotion of metastasis by the presence of VEGFR1$^+$ cell clusters. This difference in metastatic outcome as compared with previously published reports[15, 17] may be due to differences in the mouse strain used, but if these two related cell lines show a similar pattern of metastasis-promotion or -inhibition when grown in the same mouse strain then they present a great opportunity. Given the similar heritage of these cell lines, direct comparison of gene expression patterns could provide great insight into the factors essential for either concomitant tumor resistance and/or pre-metastatic niche formation. Additionally, investigation of the effect of a primary tumor on secondary metastasis should be investigated using primary tumors that have both high and low OPN expression. The MDA-MB-435 cells would be a valuable model in this regard, as it has been shown that shOPN transfection results in complete abrogation of OPN expression[41]. Additionally, these cells are known to express TF, therefore the role of hemostasis identified here would be preserved[42].

Cancer exists and progresses through interaction and manipulation of host tissues, systems and responses. Only through understanding the effects of a developing malignancy on normal host biology will the process of metastasis be thoroughly understood. This understanding will provide insight into new avenues of treatment and will significantly improve disease management and disease outcome.

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Appendices

Appendix A: Copyright Agreements and Approvals for Previously Published Work

Correspondence regarding copyright for Chapter 2, published as “Effect of anti-fibrinolytic therapy on experimental melanoma metastasis” by JM Kirstein, KC Graham, LT MacKenzie, DE Johnston, LJ Martin, AB Tuck, IC MacDonald, and AF Chambers in Clinical and Experimental Metastasis 2009;26(2):121-31.

Jenn Kirstein **Fri, Feb 18, 2011 at 2:30 PM**

To

Hello Dr Ruiz,

I have an article published in the journal 'Clinical and Experimental Metastasis' and am seeking permission to use that article as part of my PhD thesis.

The article is "Effect of anti-fibrinolytic therapy on experimental melanoma metastasis, Kirstein JM, Graham KC, Mackenzie LT, Johnston DE, Martin LJ, Tuck AB, MacDonald IC, Chambers AF., Clin Exp Metastasis. 2009;26(2):121-31".

Thank you,

Jenn

Jennifer Kirstein
PhD candidate
Department of Medical Biophysics
University of Western Ontario
London Ontario Canada

Ruiz, Melania, Springer SBM NL **Mon, Feb 21, 2011 at 2:21 AM**

To: Permissions Europe/NL

Cc: Jenn Kirstein

Dear Colleague,

Could you please help Jennifer with her below request?

Thank you,

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Tue, Feb 22, 2011 at 6:17 AM

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Correspondence regarding text chapter entitled: Interactions of Normal Tissues and Systems with Metastatic Cells: Impact on Location, Survival and Growth" to be included in "Experimental and Clinical Metastasis: A Comprehensive Review", Springer, In Press.

From: Ann Chambers
Sent: February 17, 2011 4:01 PM
To: Julia Burnier
Subject: RE: RE: Book Chapter

Hi Julia -

Any further word on this chapter or the correct citation.

Jenn is in the process of writing up her PhD thesis and would like to list this as an in press citation, if possible.

Thanks.
Ann

From: Julia Burnier
Sent: February 18, 2011 7:10 AM
To: Ann Chambers
Cc: Miguel N. Jr. Burnier, Dr.; Tiffany Porraccio
Subject: RE: RE: Book Chapter

Dear Dr. Chambers,

Since our last contact, I have moved from the Henry C. Witelson Laboratory to Spain to pursue my postdoc. I therefore CC-ed all those with the information regarding the book, the citation, and any other future information you may need regarding our collaborative book. Tiffany, could you kindly ask Springer for the correct citation and send it to Dr. Chanbers?

I think it is ok for Jenn to cite the book as "in press" since it was a request from Springer and is currently being edited by them. Tiffany, perhaps you can double check this as well.

Please send my congratulations to Jen on finishing her degree.

With warm regards
Julia

From: Tiffany Porraccio
To: Ann Chambers
Date: Mon, 21 Feb 2011 11:19:09 -0500
Subject: RE: RE: Book Chapter
Dear Dr. Chambers,

I will be in contact with all the authors this week to give them an update on the book and how to correctly cite it. As for an update, we have submitted the chapters to Springer and will finalize soon. The correct citation is "Experimental and Clinical Metastasis: A Comprehensive Review - In Press (Springer)", as was suggested by Julie, it is correct to write In Press after the title.

If you need anything else, please do not hesitate to contact me.

Best regards,
Tiffany

Tiffany Porraccio
Research Assistant
Henry C. Witelson Ocular Pathology Laboratory
3775 University, Room 216
Montreal (Quebec) Canada H3A 2B4

Appendix B: Animal Experimentation Ethics Approval

Western

10.01.08
***This is the 3rd Renewal of this protocol**
*A Full Protocol submission will be required in **2009**

Dear Dr. **MacDonald**

Your Animal Use Protocol form entitled:

In Vivo Video Microscopy: Identifying the steps in matastasis and therapeutic target

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **10.01.08 to 09.30.09**

The protocol number for this project remains as **2005-025**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. N Hague, W Lagerwerf

***The* University *of* Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 ● FL 519-661-2028 ● www.uwo.ca / animal

Western

10.01.10
***This is the 1st Renewal of this protocol**
*A Full Protocol submission will be required in **2013**

Dear Dr. **Chambers**

Your Animal Use Protocol form entitled:

Steps of Breast Cancer Metastasis: Experimental Models and Identification of Targets for Intervention.

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **10.01.10 to 10.01.11**

The protocol number for this project remains as **2009-072**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this *Animal Use Protocol* is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. N. Hague, T. Carter

***The* University *of* Western Ontario**
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Curriculum Vitae

Name:	**Jennifer Maclean, nee Kirstein**
Post-secondary Education and Degrees:	University of Western Ontario London, Ontario, Canada 1999-2003 BSc Honors Genetics with Distinction The University of Western Ontario London, Ontario, Canada 2003-2005 MSc Candidate, Department of Medical Biophysics The University of Western Ontario London, Ontario, Canada 2005-2011 PhD Candidate, Department of Medical Biophysics
Honours and Awards:	Breast Cancer Society of Canada Studentship, Feb 2010-Jun 2011 Breast Cancer Society of Canada Studentship, Sep 2009-Jul 2009 Graduate Student Research Grant, UWO, Dec 2008 National Cancer Institute of Canada, Terry Fox Foundation Young Investigator Travel Grant, Aug 2008 Natural Sciences and Engineering Research Council of Canada, Postgraduate Scholarship, PhD, Sep 2005-Aug 2008 Oncology Research Day Poster Award, May 2007, May 2004 Oncology Research Day Oral Presentation Award, May 2005 Natural Sciences and Engineering Research Council of Canada, Canada Graduate Scholarship, MSc, Sep 2004-Aug 2005 Breast Cancer Society of Canada Studentship, Sep 2003-Aug 2005 Ontario Graduate Scholarship, May 2003-Aug 2004 Natural Sciences and Engineering Research Council of Canada, Undergraduate Research Award, May 2002-Aug 2002 Louis H. Simmons Continuing Scholarship, Sep 2000-Apr 2003
Related Work Experience	**Business Development Specialist, London and Windsor Region MITACS** Jan 2011-Present Assisted companies and organizations in the development of new research initiatives, identified appropriate academic expertise, and facilitate partnership between industy and academic researchers.

Graduate Seminar Coordinator:
Department of Medical Biophysics
Sep 2006-May 2010
Organized graduate research seminars, including presentation schedule, room bookings, and notification of fellow students regarding presentation guidelines and submission deadlines.

Teaching Assistant:
Medical Sciences Laboratory.
Jan-Apr 2008, Jan-Apr 2009
Assisted 4th year students in laboratory course focusing on animal model of atherosclerosis.
Biological Macromolecules. Sept-Dec 2004, Sept-Dec 2005
Presented weekly lecture-style tutorial sessions on course material. Maintained course webpage with question/answer forum.

Undergraduate Supervisor:
Department of Medical Biophysics. May 2007-Apr 2008
Guided 4th year research student through project development and data interpretation to ensure fulfillment of course and graduation requirements.

Publications: (listed under maiden name 'Kirstein')

Kirstein, JM and Chambers AF. Interactions of Normal Tissues and Systems with Metastatic Cells: Impact on Location, Survival and Growth. In: Metastases: From the Bench to the Bedside, Editors: J Burnier and MN Burnier, Springer. In Press, August 2010.

Kirstein JM, Hague MN, McGowan PM, Graham, KC, Chambers AF. Primary melanoma tumor decreases metastasis through alterations in systemic coagulation. Manuscript Submitted to Cancer Research, September 2010.

McGowan PM, **Kirstein JM**, Chambers AF. Micrometastatic disease and metastatic outgrowth: clinical issues and experimental approaches. Future Oncology, 2009; 5(7):1083-98.

Kirstein JM, Graham KC, Mackenzie LT, Johnston DE, Martin LJ, Tuck AB, MacDonald IC, Chambers AF. Effect of anti-fibrinolytic therapy on experimental melanoma metastasis. Clinical & Experimental Metastasis, 2009;26(2):121-31.

Professional Development:

Public Science in Canada: Strengthening Science and Policy to Protect Canadians. May 12-14, 2010, Gatineau, Quebec.
Gained further understanding of national science policy and established contacts with federal scientists.

Student-2-Business Networking Conference. Jan 28, 2010, London, Ontario
Connected with London's business and community representatives and discovered many industry players within London.

Public Science in Canada: Strengthening Science to Protect Canadians. Sept 6-7, 2007, Gatineau, Quebec.
Attended conference to discuss the role of public science in Canada and gained insight into national science policy.

Coordinator - Careers Beyond Academia: UWO, Faculty of Medicine and Dentistry, May 2007-Apr 2008.
Spearheaded and organized the first career day for MSc and PhD students looking for career opportunities outside of academia.
Moderated panel discussion with students and federal government representatives.