THE ROLE OF PLATELETS IN VASCULOGENIC MIMICRY

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A thesis submitted for the degree of

Doctor of Philosophy

SEPTEMBER 2017

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Abbreviations

3D	3-dimensional
7-AAD	7-aminoactinomycin D
ACTB	beta (β)-actin gene
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ANAX2	annexin II
B16/F10	mouse melanoma cell line
BSA	bovine serum albumin
C32	human melanoma cell line
CCLE	cancer cell line encyclopedia
CD	cluster of differentiation
CD31	platelet endothelial cell adhesion molecule
CD41	integrin alpha-IIb
CD42b	platelet glycoprotein Ib alpha chain
CD62P	p-selectin
cDNA	complementary deoxyribonucleic acid
CHL-1	human melanoma cell line
CM	Tumour-conditioned platelet medium
COX-1	cyclooxygenase 1

COX-2	cyclooxygenase 2
CYCA	cyclin A
DAB	3,3′-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
EphA2	Ephrin type-A receptor 2
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylene diamine tetra acetic acid
ER	Estrogen receptor
FII	Prothrombin (coagulation factor II)
FIII	Tissue factor (coagulation factor III)
FACS	fluorescence activated cell sorting
FAK	Focal adhesion kinase
FBS	fetal bovine serum
FFPE	formalin-fixed paraffin embedded
FOV	field of view

FSC	forward scatter
Fwd	Forward
GAPDH	glyceraldehyde 3-phosphate dehydrogenase gene
GPIIb/IIIa	glycoprotein IIb/IIIa, platelet receptor
H&E	haematoxylin and eosin
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HER2	human epidermal growth factor receptor 2
HIF	hypoxia-inducible factor
HUVEC	human umbilical vein endothelial cell
HX	Haematoxylin
IHC	immunohistochemistry
IMVS	Institute of Medical and Veterinary Science
ITGAV	integrin α*v* subunit encoding gene
ITGB3	integrin β3 subunit encoding gene
KD	knockdown
KDR	vascular endothelial growth factor receptor 2
LAMC2	laminin subunit gamma 2 gene
LLC	lewis lung carcinoma
MAPK	mitogen-activated protein kinase

MDA-MB-231	human breast adenocarcinoma cell line
MDA-MB-232-LM2	MDA-MB-231 derivative
MFI	mean fluorescence intensity
MFP	mammary fat pad
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MVD	microvascular density
NCBI	National Center for Biotechnology Information
NCL	Nucleolin
NIH	US units
N.S.	not significant
NK cells	natural killer cells
NOD/SCID	non-obese diabetic/severe combined immunodeficiency
NSAID	nonsteroidal anti-inflammatory drug
p/s	photons per second
PAS	Periodic acid Schiff
PAR-1	proteinase-activated receptor-1
PAR-4	proteinase-activated receptor-4
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PG	prostaglandin
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PR	progesterone receptor
RGD	arginine-Glycine-Aspartic acid
RT-qPCR	quantitative reverse transcription PCR
RBC	red blood cell
Rev	Reverse
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	room temperature
SELPLG	p-selectin glycoprotein ligand-1 encoding gene
SEM	standard error of the mean
SHP-1	Src homology region 2 domain-containing phosphatase-1
siRNA	small interfering RNA
SSC	side scatter
TBS	tris-buffered saline
TGF-β	Transforming growth factor-beta
TNBC	triple-negative breast cancer
TSP-1	thrombospondin 1

TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
TXA_2	thromboxane A2
VE-Cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGR2	vascular endothelial growth factor receptor 2
vWF	von Willebrand Factor
VM	vasculogenic mimicry
WBC	white blood cell

Publications and conference proceedings

Published article: Tan, LY, **Martini, C**, Fridlender, ZG, Bonder, CS, Brown, MP & Ebert, LM 2017, 'Control of immune cell entry through the tumour vasculature: a missing link in optimising melanoma immunotherapy?', Clin Transl Immunology, vol. 6, no. 3, Mar, p. e134.

Oral presentation: **Martino, C**, Ebert, LM, Dale, BJ, Woods, AE & Bonder, CS, 'Platelets and vasculogenic mimicry by tumour cells', *Gordon Research Seminar*, (April 2015), **Lucca, Tuscany, Italy**

Poster presentation: **Martino, C**, Ebert, LM, Dale, BJ, Woods, AE & Bonder, CS, 'Platelets and vasculogenic mimicry by tumour cells', *Gordon Research Conferences*, (April 2015), **Lucca, Tuscany, Italy**

Poster presentation: **Martino, C**, Ebert, LM, Dale, BJ, Woods, AE & Bonder, CS, 'Platelets and vasculogenic mimicry by tumour cells', *Australian Society for Medical Research, South Australian Scientific Meeting*, National Wine Centre, (June 2015), **Adelaide, SA, Australia.**

Abstract

As with all living tissues, solid tumours must obtain a blood supply to gain access to the oxygen and nutrients required for survival and growth. This is achieved in several ways including through sprouting angiogenesis (the formation of endothelial-cell (EC)-lined vessels formed from pre-existing host vessels), co-option of existing vessels and vasculogenic mimicry (VM) among others. In VM, aggressive tumour cells that produce their own basement membrane arrange into vascular-like structures which anastomose with the host vasculature and have the capacity to carry blood towards the tumour. Studies have highlighted a current unmet need to target not only angiogenesis but also EC-independent pathways such as VM.

Platelets are small megakaryocyte fragments which, while essential in maintaining haemostasis, are also known to be abundant carriers of both pro- and anti-angiogenic components. Platelets play a role in tumour angiogenesis by supporting the development of EC-lined tumour angiogenic vessels, despite their ability to also release anti-angiogenic compounds. Although many studies have investigated the role of platelets in cancer, it is currently unknown whether platelets are also involved in regulating VM by cancer cells.

The current study addressed this question by specifically examining the effect of platelets on VM both *in vitro* and *in vivo*, as well as elucidating the mechanisms involved. Investigations were conducted using *in vitro* assays followed by an *in vivo* model of triple-negative breast cancer (TNBC) in which both normal platelets and those with a functional defect (aspirin administration) were examined. An *in vitro* tube formation assay was utilised, as well as protein and molecular assays to investigate the role of platelets in VM.

Platelets inhibited VM formation by breast cancer and melanoma cell lines *in vitro* through direct interaction rather than simple spatial interference. The inhibitory effect of platelets on VM was selective in the breast cancer and melanoma cell lines tested, since not all cancer cell lines responded in the same manner. Further to this, VM was also inhibited by contents released from α-thrombin-activated platelets. Time lapse analysis indicated that platelets inhibited the final stage of melanoma cell VM formation in the C32 cell line but not the CHL-1 cell line *in vitro*. Molecular and protein studies showed that the selective platelet inhibitory effect on VM was associated with cancer phenotype and the expression of cancer cell surface markers (expression of *SELPLG* and *ITGB3*). In addition, the CD36/thrombospondin-1 signalling was identified as a novel pathway in VM.

While the *in vivo* study found that aspirin treatment had no effect on VM formation, it did inhibit tumour burden and induced elevated tumour necrosis. In addition, aspirin treatment elevated matrix metalloprotease (*MMP)2* and *MMP9* gene expression suggesting an increased ability of cancer cells to remodel the extracellular matrix (ECM). Although conclusions cannot be made regarding the role of platelets in VM *in vivo*, an inhibitory role *in vitro* was found. This is the first report to demonstrate an involvement of platelets in VM formation.

Declaration

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged.



Carmela Martino

Dedication

To my family, you never stopped believing in me and have loved me unconditionally.

Acknowledgements

I firstly want to express many thanks to my supervisors Associate Professor Claudine Bonder, Associate Professor Anthony Woods, Dr Lisa Ebert, and Dr Brian Dale. Thank you for always believing in me especially in the most difficult times throughout this challenging project. Thank you for the guidance and constant support you provided me with, I would have not made this great achievement without all of you.

I would like to also thank the lab members of the Vascular Biology and Cell trafficking laboratory, you were always ready to help every time I popped my head in. Thank you to all of you; Sam, Minky, Lih, Emma, David, Eli, Kate, Jake, Kay, Tash, Mark and Zaheid. A special thanks to Emma who assisted me in more than one area, especially throughout the animal studies and genetic analysis. Thank you, Sam, for assisting me with the immunohistochemistry, for the comforting chats and for believing in me. Thank you, Tash, for assisting me with the TUNEL staining and imaging, and to Associate Professor Michael Samuel for enabling me to use his facilities.

A special thank you to the girls at UniSA. First and foremost, Jess, thank you for being there for me throughout it all. You have one of the kindest hearts I have ever known. Thank you also to Rachael, Bek, Sophie, Steph and Melissa. You have all been there for me and picked me up when I struggled. Thank you for all the times you helped me, my darling friends.

I would also like to thank Rupal and Andrew, you provided me with so much love and kindness throughout my PhD. Thank you for the help you have given me and for the comforting chats. Thank you for believing in me every step of the way. I will forever treasure your friendship.

Furthermore, I would like to thank the Australian Government and the University of South Australia for the Australian Postgraduate Award and UniSA scholarships through the Research Training Program which has allowed me to undertake my studies.

I would like to give a special and heartfelt thank you to my family. Mum and dad, you never stopped believing in me and your love made me keep going. Thank you for the sacrifices you made for me throughout it, and for loving me unconditionally. Thank you, Enzo and Cosi, you've been the best brothers I could ever ask for. Thank you also to my Nonna Orsola and late Nonno Pasquale. To my mother-in-law and father-in-law, Ana and Joe, thank you for believing in me and supporting me. I love you all from the bottom of my heart.

Finally, I would like to give a warm and special thank you to my husband, Rimo. Thank you, my love, for being my rock and supporting me throughout the challenges during my PhD. You never stopped believing in me. Thank you for picking me up in the worst of times, and for giving me so much encouragement. Thank you for everything you have done for me, I love you immensely, words cannot express.

Chapter 1. Introduction

1.1 Introduction overview

Globally, cancer is a leading cause of death and in 2015 was responsible for a total of 8.8 million deaths, 16% of the world's population (World Health Organization [WHO] 2015). Breast cancer and melanoma are amongst the leading causes of death in Australia and worldwide (WHO 2015). It was estimated by the WHO that breast cancer alone, the most common cancer in women, resulted in the death of over 560,000 women worldwide in 2015, while malignant melanoma was the cause of over 61,000 deaths worldwide (WHO 2015).

Platelets are recognised as having primarily a promotive role towards tumour angiogenesis and metastasis (Hara, Steiner & Baldini 1980; Sabrkhany, Griffioen & Oude Egbrink 2011), with elevated numbers correlating with a poor prognosis (Ikeda et al. 2002; Pedersen & Milman 1996; Shimada et al. 2004; Sirott et al. 1993). Vasculogenic mimicry is also well recognised as a major contributor to the development of cancer (Hendrix et al. 2003a; Maniotis et al. 1999; Vartanian 2012). However, there are no studies to date that have investigated the role of platelets in VM.

This chapter will begin by highlighting the structure of platelets followed by an introduction into the physiological importance of platelets, detailing their participation in haemostasis and angiogenesis. A detailed discussion into the pathological impact of platelets in cancer will then ensue, focussing on their role in tumour angiogenesis and metastasis. Vasculogenic mimicry (VM) will be introduced followed by a detailed and critical analysis of the process, including morphological and phenotypic characteristics, the clinical impact in cancer, and signalling pathways involved. Finally, the end of this chapter will highlight gaps in the literature by discussing current anticancer therapeutics, with a focus on VM targeting strategies, and provide evidence suggesting the involvement

of platelets in VM. The hypothesis driving this thesis and the general aims to be addressed will be stated.

1.2 Platelet structure

Platelets, also commonly referred to as thrombocytes, are small (1-3μm in diameter) anucleate cellular fragments that are formed from large progenitor bone marrow cells known as megakaryocytes (Figure 1-1). Cytoplasmic components found in platelets include glycogen granules, mitochondria and three different types of secretory granules alpha (α), dense, and lysosomal) (White 2013). Platelet formation is regulated by thrombopoietin (TPO; produced primarily in the liver) which stimulates growth and maturation of the platelet-producing megakaryocytes. These form pseudopodia-like structures which elongate and become narrow to create structures known as proplatelets. The budding and fragmentation of proplatelets then leads to platelet formation (Italiano et al. 1999; Machlus, Thon & Italiano 2014; Thon & Italiano 2010). Platelet numbers in circulating blood range between 150–450x10^6 per mL and have a half-life of approximately 8-10 days in humans and 3-4 days in mice (Schmitt et al. 2001). In comparison, human platelets also have a larger mean platelet volume than murine platelets (Schmitt et al. 2001). At the end of their life cycle, platelets undergo apoptosis (regulated by pro-survival Bcl-xL, and pro-apoptotic proteins Bak and Bax) (Lebois & Josefsson 2016). Ultrastructurally, platelets are composed of an outer membrane which is connected to an open canalicular system (OCS) which is closely intertwined with the dense tubular system (Figure 1-2).

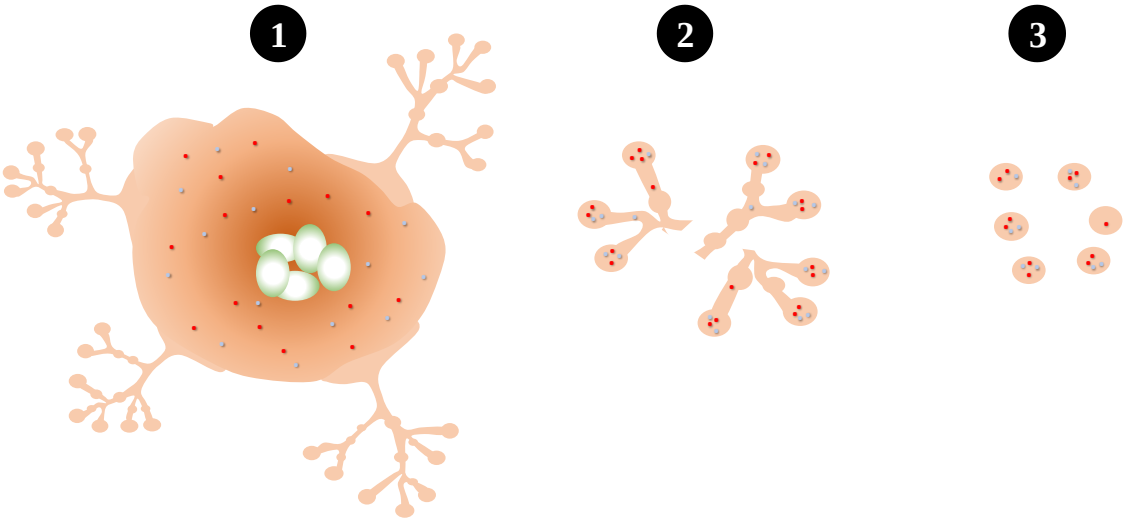


Figure 1-1. Platelet formation

In **1**; Mature megakaryocyte with α and dense granules (red and blue particles) and pro-platelet projections at the surface. In **2**; proplatelets are released from megakaryocytes. In **3**; platelets are formed via fragmentation of proplatelets.

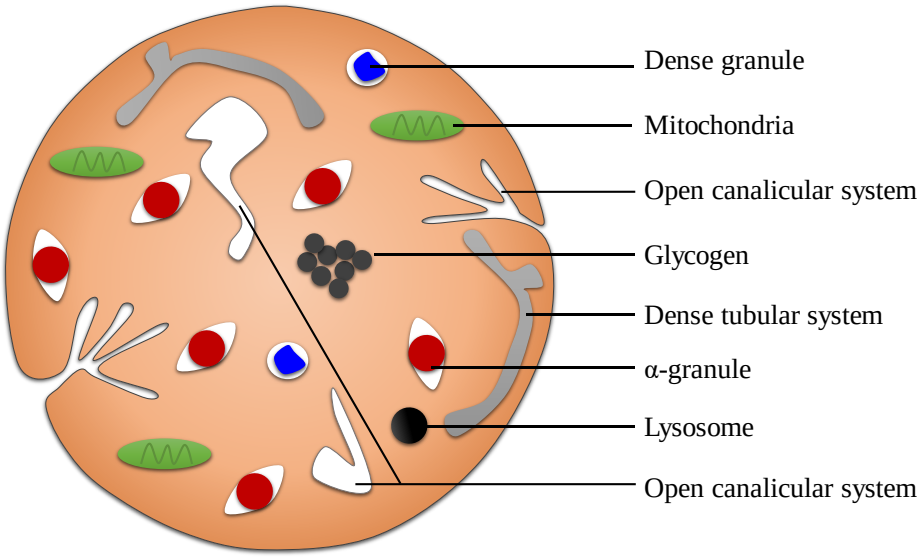


Figure 1-2. Structure of platelets

Platelets are composed of an open canalicular system (OCS) interconnected with a dense tubular system. The cytoplasm contains mitochondria, glycogen, and secretory granules (alpha (α)-granules, dense granules, and lysosomes).

1.2.1 **Platelet granules**

Platelet granules are transferred from megakaryocytes into platelets upon proplatelet formation (Italiano et al. 1999; Larson & Watson 2006; Machlus, Thon & Italiano 2014), and are released following platelet activation. Platelets consist of three types of secretory granules, namely α-, dense and lysosomal granules. The α-granules are the most abundant platelet granules, found in numbers of 50-80 granules per human platelet and are approximately 200–500nm in diameter (Blair & Flaumenhaft 2009; Harrison, Savidge & Cramer 1990). In contrast, the smaller dense (150nm) and lysosomal granules (200-250nm) are both found in small numbers: 3-8 dense granules and <3 lysosomal granules per platelet (Flaumenhaft 2013; McNicol & Israels 1999; Rendu & Brohard-Bohn 2001; Rumbaut & Thiagarajan 2010).

1.2.1.1 ***Alpha granules***

Alpha granules are exclusive to platelets and are equivalent to Weibel-Palade bodies in endothelial cells (ECs). In platelets, alpha granules are the most common granule type and also contain the majority of the molecules which are released by platelets. As summarised in Table 1-1, these include haemostatic factors, angiogenic and anti-angiogenic factors, proteases, matrix proteins, proteases and inhibitors, and inflammatory cytokines and chemokines (Coppinger & Maguire 2007; Gear & Camerini 2003; Gleissner, von Hundelshausen & Ley 2008; Menter et al. 2014; Peterson et al. 2010). Three-dimensional (3D) mapping from electron tomography revealed that platelet α-granules have a highly heterogeneous membrane, allowing them to be classified into three main groups (van Nispen tot Pannerden et al. 2010). These are spherical granules, multivesicular granules (containing vast number of luminal membrane vesicles), and a tubular subtype (50-nm-wide tubular granules).

Table 1-1. List of molecules within α granules in platelets

Classification	Secreted molecule
Haemostatic factors	Coagulation factors (V, XI, VIII, XIIIA chain) Fibrinogen α and γ chains Prothrombin von Willebrand factor (vWF)
Angiogenic factors	Angiogenin Angiopoietin-1 Basic fibroblast growth factor (bFGF) Epidermal growth factor (EGF) Platelet derived growth factor (PDGF) Transforming growth factor beta 1 (TGF-β1) Vascular endothelial growth factor (VEGF-A, -C) Stromal cell derived factor-1α (SDF-1α) Sphingosine-1-phosphate (S1P)
Anti-angiogenic factors	Angiostatin Endostatin Platelet factor 4 (PF4; CXCL4) Plasminogen activator inhibitor-1 (PAI-1) Thrombospondin-1 (TSP-1)
Matrix proteins, proteases and inhibitors	Fibronectin PAI-1 Tissue inhibitor of metalloproteinases (TIMP-1, 2, -4) Matrix metalloproteases (MMP1, 2, 3, 9, 14) Vitronectin
Chemokines	CCL2, CCL3, CCL5, CCL7, CCL17, CXCL1, CXCL4, CXCL5, CXCL7, CXCL8, CXCL12

Furthermore, anti-angiogenic and pro-angiogenic factors are suggested to be contained in separate α-granules which are differentially released depending on the stimulus (further details in section 1.3) (Chatterjee et al. 2011; Italiano et al. 2008). Defects in α-granules are associated with bleeding, an example of this being grey platelet syndrome wherein there is a deficiency in the number of α-granules and their contents in the platelet cytoplasm, resulting in increased bleeding tendencies in patients (Simon, Kunicki & Nugent 2008).

1.2.1.2 *Dense granules*

Like α-granules, dense granules are unique to platelets and their contents (summarised in Table 1-2) include the nucleotides adenosine diphosphate (APD) and adenosine triphosphate (ATP), guanosine diphosphate (GDP), guanosine-5'-triphosphate (GTP), bioactive amines such as serotonin and histamine, magnesium and calcium, and phosphates (Chatterjee et al. 1990; Meyers, Holmsen & Seachord 1982; Ruiz et al. 2004). Defects in dense granule release cause a spectrum of bleeding disorders similar to that in patients with grey platelet syndrome (Simon, Kunicki & Nugent 2008).

1.2.1.3 *Lysosomal granules*

Lysosomal granules are found in very low number (<3 per platelet) (Flaumenhaft 2013; Rendu & Brohard-Bohn 2001). As shown in Table 1-2, lysosomes are known to contain proteases and glycosidases (degradation enzymes for proteins, carbohydrates and lipids), although the exact role of these granules in platelet function is yet to be fully elucidated (Fitch-Tewfik & Flaumenhaft 2013).

Table 1-2. List of molecules within dense and lysosomal granules in platelets

Classification	Dense granule secreted molecules
Nucleotides	ADP
	ATP
	GDP
	GTP
Bioactive amines	Serotonin
	Histamine
Cations	Calcium
	Magnesium
Phosphates	Polyphosphate
	Pyrophosphate
	Lysosome granule secreted molecules
Acid proteases	Cathepsins
	Peptidases
	Collagenase
	Acid phosphatase
	Arylsulphatase
Glycohydrolases	Heparinase β-N-acetyl-glucosaminidase, β-glucuronidase, β-galactosidase, β-glycerophosphatase, α-D-glucosidase, β-D-glucosidase, α-L-fucosidase, β-D-fucosidase, α-L-arabinosidase, α-D-mannosidase

1.2.2 **Platelet receptors**

There are many proteins on the platelet surface, consisting mainly of glycoproteins, which are important for mediating adhesion between platelets and other cells including the vascular endothelial cells (ECs). Such adhesion triggers platelet shape change and activation (Saboor et al. 2013). Several platelet receptors have been reported to be important for aggregation and activation, including integrins, leucine-rich repeat receptors, selectins, tetraspanins, transmembrane receptors, prostaglandin receptors, lipid receptors, immunoglobulin superfamily receptors and tyrosine kinase receptors (Clemetson & Clemetson 2013; Kasirer-Friede, Kahn & Shattil 2007; Kauskot & Hoylaerts 2012; Saboor et al. 2013). Whilst these receptors are important in normal haemostatic function of platelets, there is also evidence to support their involvement in pathological conditions including tumour growth and metastasis (Bambace & Holmes 2011; Erpenbeck & Schon 2010; Sabrkhany, Griffioen & Oude Egbrink 2011). The major receptors important in the adhesive and activation properties of platelets are described below and illustrated in Figure 1-3.

1.2.2.1 ***Glycoprotein receptors and complexes***

Glycoprotein (GP)IIb/IIIa receptor (also known as integrin $\alpha_{IIb}\beta_3$) is composed of integrin subunits αIIb and β_3 and is the most abundant receptor found on the platelet surface (80,000-100,000 copies per platelet) (Bennett 2017; Burkhart et al. 2012). GPIIb/IIIa is a receptor for the arginine-glycine-aspartic acid (RGD) binding site within fibrinogen, fibrin, von Willebrand factor (vWF), thrombospondin-1 (TSP-1), fibronectin and vitronectin (Figure 1-3) (Bennett 2017; Kasirer-Friede, Kahn & Shattil 2007; Weisel et al. 1992). Platelets can mediate adhesion to other platelets and other cells through the

formation of RGD bridges between GPIIb/IIIa and integrin β_1, β_3, and β_5 family members expressed by other cell types.

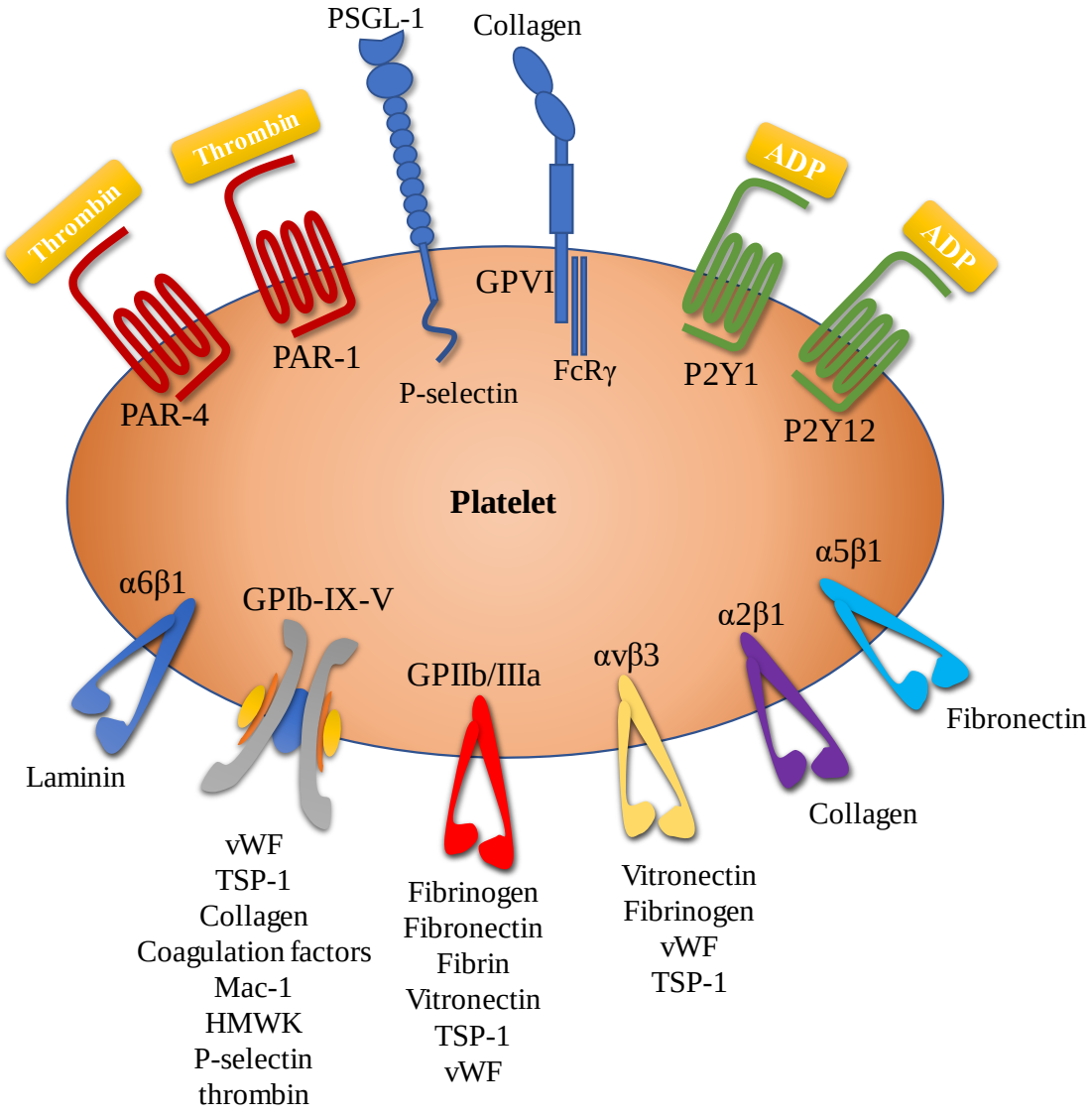


Figure 1-3. Surface receptors of platelets

The figure illustrates the major platelet receptor-ligand interactions which mediate the haemostatic function of platelets. **ADP**, adenosine diphosphate; **FcRγ**, Fc receptor common γ-chain; **HMWK**, high-molecular-weight kininogen; **Mac-1**, macrophage-1 antigen; **P2Y**, purinergic receptor; **PAR**, protease activated receptor; **PSGL-1**, p-selectin glycoprotein ligand-1; **TSP-1**, thrombospondin-1; **vWF**, von Willebrand factor.

Thrombin activates platelets to induce shape change and trafficking of GPIIb/IIIa from internal membranes (α- and dense granules, and the OCS) to the platelet surface (Kasirer-Friede, Kahn & Shattil 2007). The GPIIb/IIIa receptor allows for the binding of fibrinogen and reinforcement of the platelet haemostatic plug (Bennett 2017; Fullard 2004; Jurk & Kehrel 2005).

GPVI is a platelet expressed receptor for collagen, fibrin and laminin which allows for platelet interaction with the extracellular matrix (ECM) (Figure 1-3). It is expressed exclusively on mature megakaryocytes (and platelets) where it associates with the transmembrane adapter protein FcRγ, and mediates collagen induced platelet activation (Kasirer-Friede, Kahn & Shattil 2007). Signalling through GPVI is documented to induce activation of platelet receptor GPIIb/IIIa (Kasirer-Friede, Kahn & Shattil 2007; Nakamura et al. 1999).

The GPIb-IX-V complex, or GPIb receptor, is another key receptor in platelet adhesion and is composed of GPIbα (also known as CD42b), GPIbβ, GPIX, and GPV subunits (Yip et al. 2005). The principal ligand for GPIb at sites of vascular injury is vWF, although several other ligands exist including TSP-1, collagen, P-selectin, kininogen, Factor XII, Factor VIIa, Factor XI, thrombin, high molecular weight kininogen, and $\alpha_M\beta_2$ (Mac-1) (reviewed in Canobbio, Balduini & Torti 2004). Binding of GPIb to vWF can mediate tethering and platelet adhesion at high shear stress along the damaged vascular wall, and lead to activation of GPIIb/IIIa. This subsequently allows for platelet aggregation to occur via GPIIb/IIIa binding to soluble fibrinogen or vWF (Yip et al. 2005). The engagement of GPIb receptor also activates several platelet integrins ($\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$) which support platelet aggregation (Yip et al. 2005).

1.2.2.2 *Integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, $\alpha v\beta_3$*

Platelet tethering and rolling along the vascular endothelium is mediated by various integrins on the platelet surface which permit interaction with exposed ECM components (Bennett, Berger & Billings 2009; Nieswandt, Varga-Szabo & Elvers 2009). Platelets are known to express several integrins incorporating β_1, or β_3 which, following activation, change from a low-affinity to a high-affinity state allowing for effective binding to their ligands (Kasirer-Friede, Kahn & Shattil 2007; Nieswandt, Varga-Szabo & Elvers 2009; Saboor et al. 2013). As illustrated in Figure 1-3, platelets express three integrin complexes of the β_1 family; $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$. Integrin $\alpha_2\beta_1$ (GPIa/IIa), in addition to GPIV (see section 0), is another receptor which binds collagen and mediates firm attachment of platelets to collagen (Bergmeier & Hynes 2012; Kasirer-Friede, Kahn & Shattil 2007; Saboor et al. 2013). This receptor serves to stabilise the haemostatic clot and promote pro-coagulant activity. Integrin $\alpha_5\beta_1$ binds fibronectin and is documented to support platelet adhesion under static conditions in the absence of platelet activation (Kasirer-Friede, Kahn & Shattil 2007). Integrin $\alpha_6\beta_1$ binds laminin, which is found in the basement membrane and ECM. Binding through this integrin does not induce P-selectin exposure or platelet aggregation, but does result in platelet morphological changes (Kasirer-Friede, Kahn & Shattil 2007).

Two integrin β_3 complexes are expressed by platelets (Figure 1-3). GPIIb/IIIa is the most abundant (80,000-100,000 copies per platelet) of all integrins (Burkhart et al. 2012), and the most studied (see section 0). This integrin is expressed on the α and dense granules and on the OCS membrane. In its high affinity state GPIIb/IIIa binds to its ligands (fibrinogen, fibrin, vWF, fibronectin, vitronectin, and TSP-1) to promote platelet aggregation (Bonnefoy et al. 2001; Kasirer-Friede, Kahn & Shattil 2007). Ligand binding to GPIIb/IIIa is known to facilitate strong adhesion and platelet spreading on ECM, fibrin

clot retraction, and promoting the pro-coagulant activity of the platelet (Kasirer-Friede, Kahn & Shattil 2007). Platelets also express integrin $\alpha_v\beta_3$, which binds to vitronectin, fibrinogen, vWF and TSP-1 in an RGD dependent manner (Kasirer-Friede, Kahn & Shattil 2007; Kauskot & Hoylaerts 2012).

1.2.2.3 *Thrombin receptors (PAR-1 and PAR-4)*

Thrombin is a serine protease product of the coagulation cascade (Figure 1-5). It is an important regulator in vascular biology as it is known to regulate platelet aggregation and activation (Burkhart et al. 2012; Coughlin 2000; Palta, Saroa & Palta 2014). The actions of thrombin are mediated via activation/ligation of protease-activated receptors (PAR) which are G-protein coupled seven-transmembrane receptors (GPCR) (Coughlin 2000; Kahn et al. 1999). Platelets express both PAR-1 and PAR-4 on the platelet membrane (Figure 1-3) and these mediate thrombin activation, granule secretion and platelet aggregation (Coughlin 2000; Kahn et al. 1999). While PAR-1 is a high-affinity receptor for thrombin, PAR-4 has low affinity for thrombin and can be activated in the absence of PAR-1 (Andersen et al. 1999; Brass et al. 1992; Hung et al. 1992; Kahn et al. 1999).

1.2.2.4 *Adenosine diphosphate (ADP) receptors ($P2Y_1$ and P2Y12)*

Although adenosine diphosphate (ADP) is not a strong platelet aggregating agent (Table 4-1), it plays an important role in haemostasis and thrombosis. ADP is stored in high quantities in the dense granules of platelets and is released following platelet activation (Coppinger et al. 2004). The procoagulant actions of ADP are known to be mediated through two different purinergic seven-transmembrane domain GPCRs on the surface of platelets (Figure 1-3), $P2Y_1$ (Gq-coupled) and $P2Y_{12}$ (Gi-coupled) (Cattaneo 2013; Leon et al. 2003; Offermanns 2006). While P2Y1 can trigger platelet aggregation, it is not

adequate for a full platelet response which is completed and amplified through the $P2Y_{12}$ receptor (Gachet 2001).

1.2.2.5 *P-selectin*

P-selectin (CD62P), a c-type lectin, is a very important receptor which is expressed in both platelets (stored in the α-granules) and ECs (in the Weibel–Palade bodies). P-selectin plays many roles in platelets including mediation of platelet-EC interactions, platelet-leukocyte tethering to the EC surface and leukocyte activation (Evangelista & Smyth 2013). Ligands for P-selectin include mucin-like P-selectin glycoprotein ligand (PSGL)-1, GPIbα, and tissue factor (TF) (Evangelista & Smyth 2013; Kauskot & Hoylaerts 2012; Romo et al. 1999). P-selectin is expressed on the surface of activated platelets (Figure 1-3) following translocation from the α-granules, and soluble P-selectin is released into plasma following platelet activation (Michelson et al. 1996; Semenov et al. 1999). It allows for platelet-platelet adhesion as well as platelet adhesion with other cells including ECs, leukocytes and also cancer cells through their expression of PSGL-1 (Frenette et al. 2000; Gong et al. 2012b; Ma, Plow & Geng 2004). When P-selectin is expressed, this results in further recruitment of platelets to tether via GPIb-V-IX mediated interactions (Furie, Furie & Flaumenhaft 2001).

1.3 Platelet activation and regulated release of granule contents

Platelet activation induces shape change as well as the fusion of platelet granules (which are normally distributed evenly throughout the non-activated platelets) with the OCS, which releases granule contents into the extracellular environment (Flaumenhaft 2013). This release of granule contents can be stimulated by several agonists from strong (e.g. thrombin, collagen and platelet-factor-4 [PF4]) through to weak activators (e.g. ADP and thromboxane [TX] A_2) (detailed in Table 4-1). Of these, thrombin is documented to be the most potent endogenous agonist for platelet activation and granule release (Chung et al. 2002; Davey & Luscher 1967). Indeed, upon activation with thrombin, platelets can release over 300 molecules including regulators of angiogenesis, haemostatic factors, proteases, matrix proteins, necrotic factors, and inflammatory cytokines and chemokines (summarised in Table 1-1 and Table 1-2) (Coppinger & Maguire 2007; Gear & Camerini 2003; Jurasz et al. 2003; Peterson et al. 2010; Ruiz et al. 2004; Whiteheart 2011).

It was widely assumed that various molecules are packaged indiscriminately within the granules of platelets such that α-granules harbour a variety of contents (e.g. both pro and anti-angiogenic compounds) which are released in a non-selective manner (Pilatova, Zdrazilova-Dubska & Klement 2012; White & Rompietti 2007). However, Segal and Storrie presented evidence to suggest that contents may in fact be packaged into separate granules (Sehgal & Storrie 2007). They showed that vWF and fibrinogen are differentially packaged in α-granules and may support the differential release of proteins from platelets. In similar studies, Italiano and colleagues demonstrated that platelets contain different subpopulations of α-granules wherein the pro-angiogenic vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), and anti-angiogenic factors, endostatin and PF4, are packaged into distinct granule subpopulations to allow for selective release (Italiano et al. 2008).

The presence of differential packaging supports the concept that depending on different stimuli, platelets can carefully coordinate a selective release of different compounds from their granules. Ma and colleagues demonstrated that activation via PAR-1 and PAR-4 differentially released angiogenesis promoters or inhibitors, respectively (Ma et al. 2005). More recent studies revealed that stimulation with PAR-1-activating peptide, ADP, and GPVI stimulation results in VEGF and SDF-α release, whereas stimulation with PAR-4-activating peptide induced release of endostatin and PF4 (Chatterjee et al. 2011). Similarly, Battinelli and colleagues observed that ADP stimulated the release of VEGF but not endostatin, whereas TXA_2 stimulated the release of endostatin but not VEGF (Battinelli, Markens & Italiano 2011). There is also evidence that selective granule release of contents from platelets also depends on environmental influences such as temperature or acidity (which are commonly altered in inflammation, infection, and cancer) (Etulain et al. 2011; Etulain et al. 2012). These studies demonstrate that secretion of bioactive substances from platelets is stimulus-dependent and is a carefully regulated process which is yet to be fully elucidated.

1.4 Physiological role of platelets

1.4.1 Haemostasis

The primary physiological function of platelets is in haemostasis and thrombus formation (Coller 2011; Golebiewska & Poole 2015). As illustrated in Figure 1-4, under normal conditions, ECs prevent platelet binding, and thus platelets circulate in an inactive state through the blood. Primary haemostasis is initiated when the endothelial layer is damaged, resulting in exposed sub-endothelial matrix proteins such as VWF, collagen, fibrinogen, laminin, and fibronectin (Bergmeier & Hynes 2012). Platelets tether and roll on exposed ECM components through surface expressed integrins (section 1.2.2.2) and adhere primarily through GPIIb/IIIa and GPIb-V-IX receptors (section 0). Activated GPIIb/IIIa on the platelet membrane is able to bind soluble fibrinogen which allows the subsequent recruitment of other platelets by acting as a bridge between GPIIb/IIIa molecules on platelets (Bledzka et al. 2013; Varga-Szabo, Pleines & Nieswandt 2008). Primary haemostasis also leads to the release of several coagulation factors from activated platelets including Factors V, XI, VIII, and FXIII A chain (section 1.2.1), allowing for secondary haemostasis (the coagulation cascade) to also occur. As illustrated in Figure 1-5, the activation of the coagulation cascade occurs through two pathways, the contact activation pathway (also known as the intrinsic pathway) and the TF pathway (also known as the extrinsic pathway). While the intrinsic pathway is initiated by factor XII activation on negatively charged surfaces, the extrinsic pathway is activated by TF released sites of vascular injury (Gailani & Renne 2007; McGee & Li 1991). Activation of the coagulation cascade initiates a series of reactions leading to the formation of thrombin from prothrombin, known as the common pathway. This results in the conversion of fibrinogen into fibrin strands that stabilise the primary platelet plug (Palta, Saroa & Palta 2014).

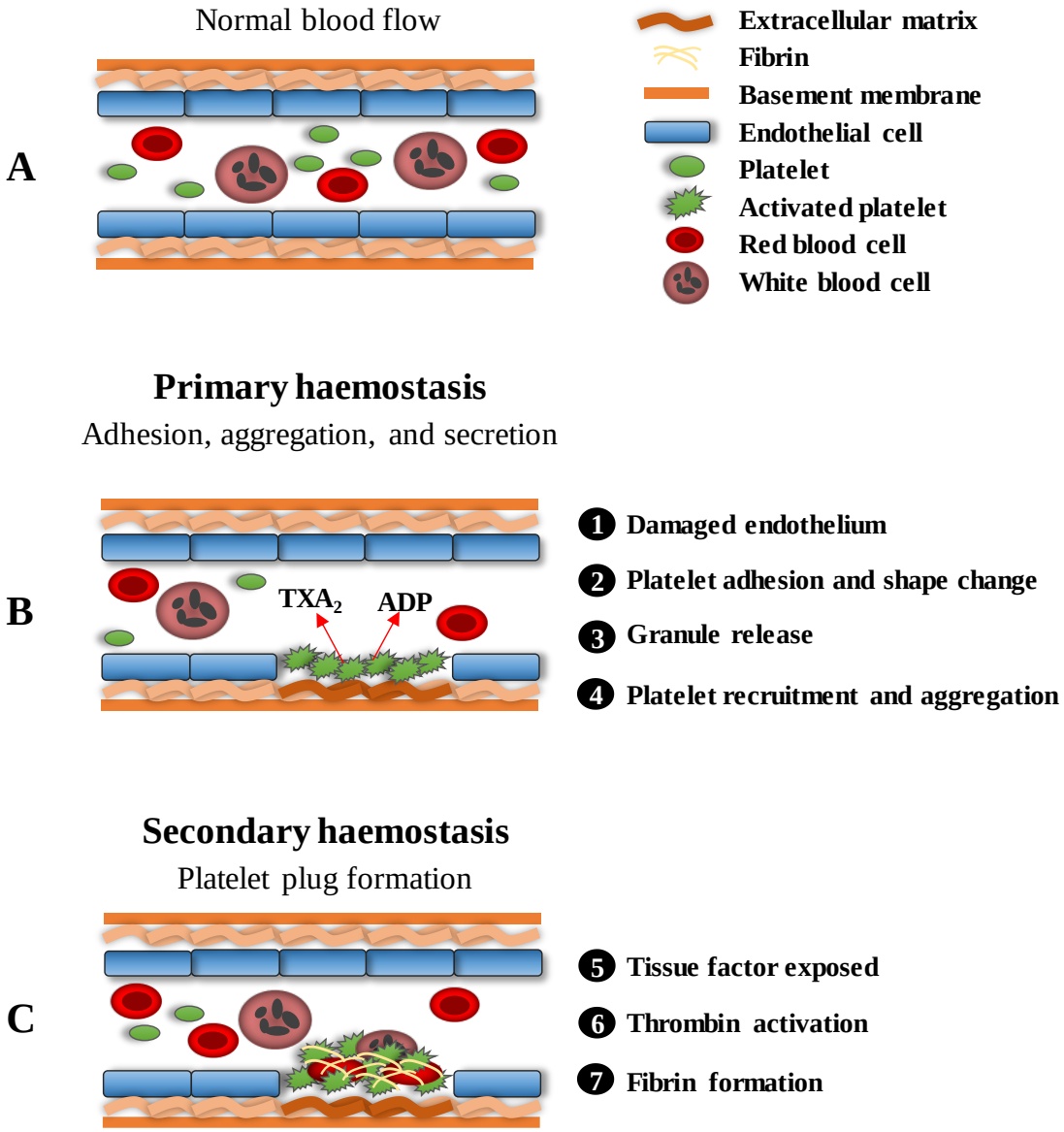


Figure 1-4. Normal blood flow and the process of primary and secondary haemostasis

(**A**) Blood flow under normal conditions. (**B**) In primary haemostasis (**1**) platelets detect exposed extracellular matrix (ECM) components under damaged endothelium, (**2**) platelets adhere which leads to platelet shape change, activation and (**3**) release of granule contents (ADP and TXA_2). (**4**) The response is amplified with the further recruitment of platelets which aggregate to form a primary platelet plug. (**C**) Secondary haemostasis is activated by (**5**) the exposure of tissue factor on the damaged endothelium which lead to (**6**) thrombin activation and finally (**7**) fibrin formation which stabilises the primary platelet plug. **ADP**, adenosine diphosphate; **TXA_2**, thromboxane A_2.

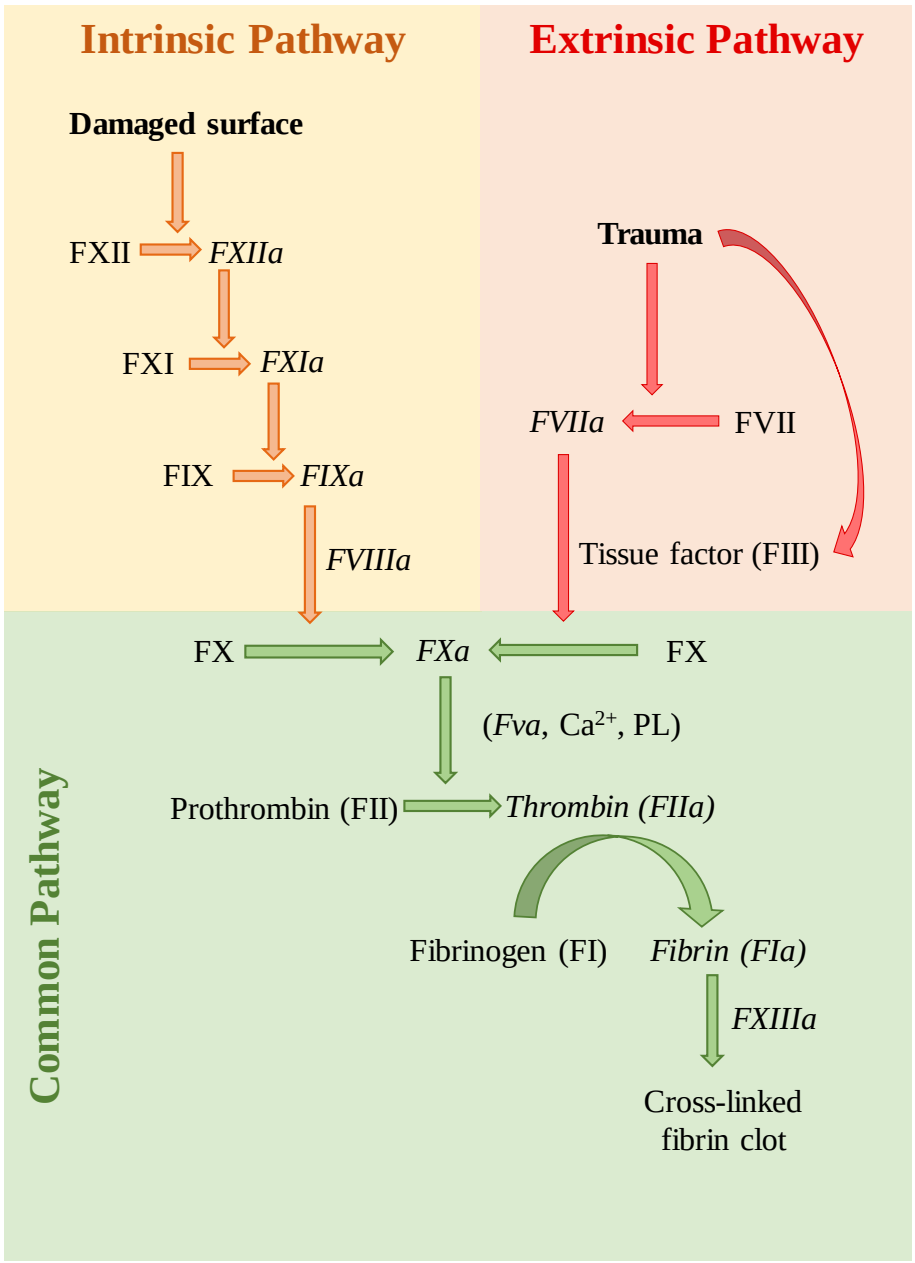


Figure 1-5. Overview of the coagulation pathway

The coagulation cascade is composed of the intrinsic (contact activation) pathway and the extrinsic (tissue factor) pathway. Both pathways involve the activation of circulating coagulation factors mediated through a series of reactions which result in activation of the common pathway (activation of FX to FXa). Prothrombin (FII) is converted to thrombin (*FIIa*) which converts fibrinogen (FI) to fibrin (*FIa*). Thrombin also activates FXIII (to *FXIIIa*) which covalently bonds fibrin strands allowing the formation of a stable fibrin clot.

1.4.2 **Angiogenesis**

1.4.2.1 ***Stages of angiogenesis***

Angiogenesis is the growth of new blood vessels from pre-existing blood vessels i.e. capillaries and post-capillary venules (Folkman 1971; Risau 1995). It is an important process in vascular development in the foetus, while in the adult it is activated in the female reproductive cycle, placenta formation, and wound healing (Carmeliet 2005; Reynolds, Killilea & Redmer 1992; Tonnesen, Feng & Clark 2000). Angiogenesis is a complex multistep process consisting of several stages and is illustrated in Figure 1-6. These including; (1) initiation, (2) an extension stage (EC proliferation, migration and differentiation), and finally (3) vessel stabilisation (lumen formation and maturation) (Adams & Alitalo 2007; Mantzaris, Webb & Othmer 2004). Angiogenesis is initiated by proangiogenic factors which activate ECs, leading to degradation of the surrounding ECM. Selected ECs known as tip cells (located at the leading edge of a sprout) alter their phenotype to obtain more invasive and motile properties and guide the growing sprout towards the angiogenic stimulus (e.g. VEGF) (Gerhardt et al. 2003). The stalk cells then follow behind tip cells and adopt a more proliferative phenotype and allow the sprouts to elongate while laying down a basement membrane (Ausprunk & Folkman 1977; Ribatti & Crivellato 2012). When tip cells encounter other sprouts, the motile and invasive properties are supressed, tubular structures anastomose and a more quiescent phenotype that promotes vessel maturation is activated, resulting in lumen formation, mural cell recruitment (e.g. pericytes and vascular smooth muscle cells), and ECM protein deposition (Flamme, Baranowski & Risau 1993; Francavilla, Maddaluno & Cavallaro 2009).

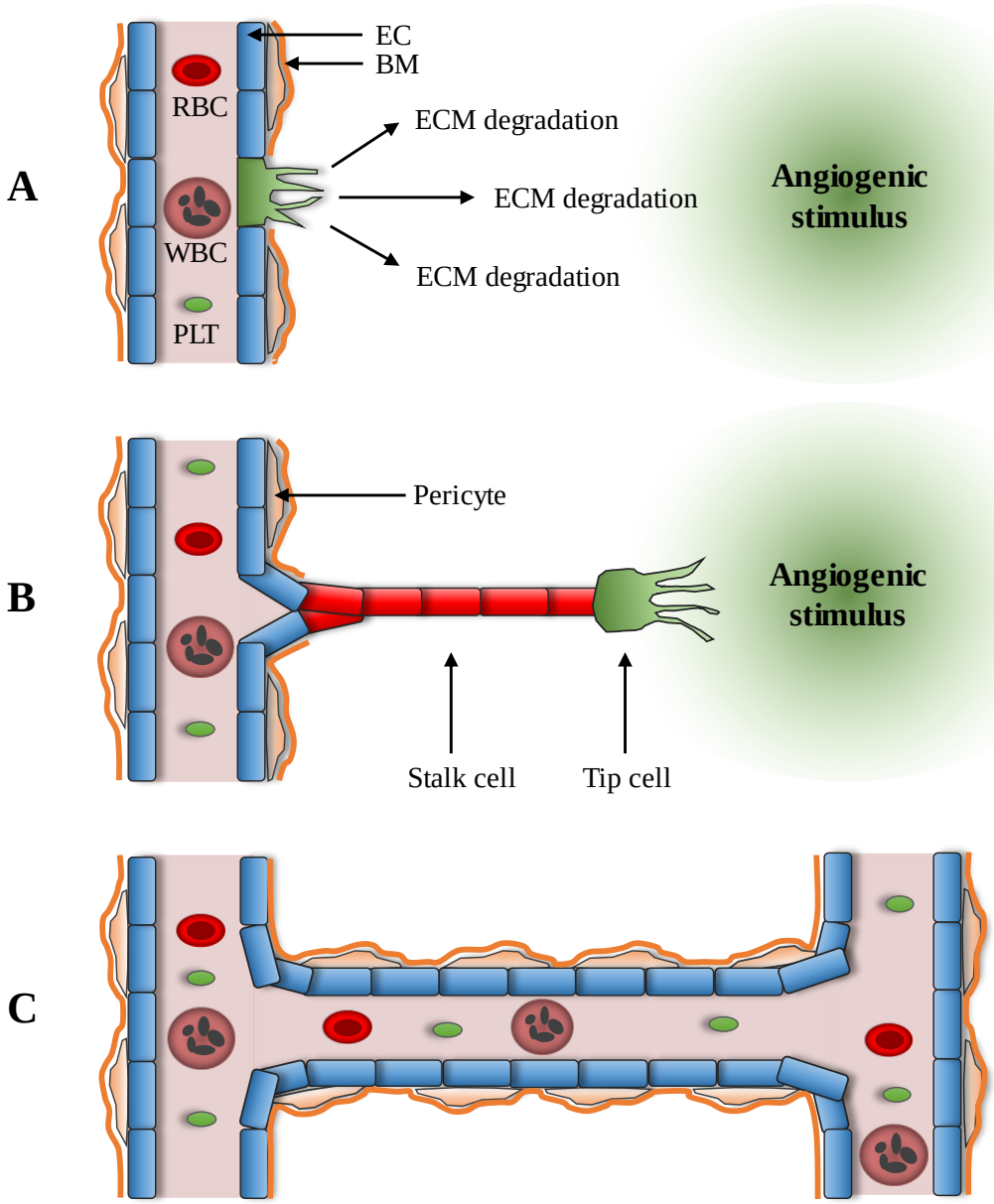


Figure 1-6. The stages of angiogenesis

In the first stage of angiogenesis (**A**), an angiogenic stimulus activates a selected EC cell into a tip cell (green) which degrades the basement membrane (BM) and extracellular matrix (ECM). In the second stage (**B**), the tip cell proliferates, migrates towards the angiogenic stimulus and ECs following the tip cell differentiate into stalk cells. In the final stage (**C**) the tip cell encounters another angiogenic sprout which inhibits its motile properties. The newly formed sprout undergoes maturation leading to lumen formation and recruitment of supporting mural cells (pericytes and vascular smooth muscle cells). **PLT**, platelet; **RBC**, red blood cell; **WBC**, white blood cell.

1.4.2.2 *Regulating factors of angiogenesis*

The process of angiogenesis is complex and several regulators have been identified, including VEGF, bFGF, angiopoietins (Ang-1 and Ang-2), platelet-derived growth factor (PDGF), transforming growth factors (TGF-β), and the negative regulator TSP-1 (Battegay et al. 1994; Ferrari et al. 2009; Hoeben et al. 2004; Koblizek et al. 1998; Lobov, Brooks & Lang 2002; Presta et al. 2005; Sato et al. 1993). By far the most important regulator of angiogenesis is VEGF-A, belonging to the VEGF family which also consists of VEGF-B, C, D, E and F, and placental growth factor (PlGF) (Ferrara 2004; Yamazaki et al. 2003). It is documented that VEGF-A signals through its receptors VEGF receptor 1 (VEGFR1; also known as Fms-like tyrosine kinase-1, Flt-1) and VEGFR2 (denoted human kinase insert domain receptor (KDR) in humans; and Flk-1 in mouse) (Figure 1-7) (de Vries et al. 1992; Terman et al. 1994). In addition, VEGF-B and PlGF are also ligands for VEGFR1, while proteolytically processed VEGF-C/D, VEGF-E and VEGF-F are ligands for VEGFR2 (reviewed in Cebe-Suarez, Zehnder-Fjallman & Ballmer-Hofer 2006; Shibuya & Claesson-Welsh 2006). Several *in vivo* studies have highlighted the importance of the VEGF signalling pathway in angiogenesis, demonstrating that mutations in the VEGF gene and its receptors, VEGFR1 and VEGFR2, results in embryonic lethality due to multiple defects in vascular structure formation (Carmeliet et al. 1996; Ferrara et al. 1996; Fong et al. 1995; Shalaby et al. 1995).

All of the above mentioned regulators of angiogenesis (VEGF, bFGF, PDGF, Ang-1 and Ang-2, TGF-β, and TSP-1) are contained in and can be secreted from the α-granules of platelets (Blair & Flaumenhaft 2009; Peterson et al. 2010). Furthermore, it has been documented that platelets are the major source of VEGF in blood (Hashiguchi et al. 2000; Mohle et al. 1997) and this has prompted many to investigate the role of platelets in angiogenesis.

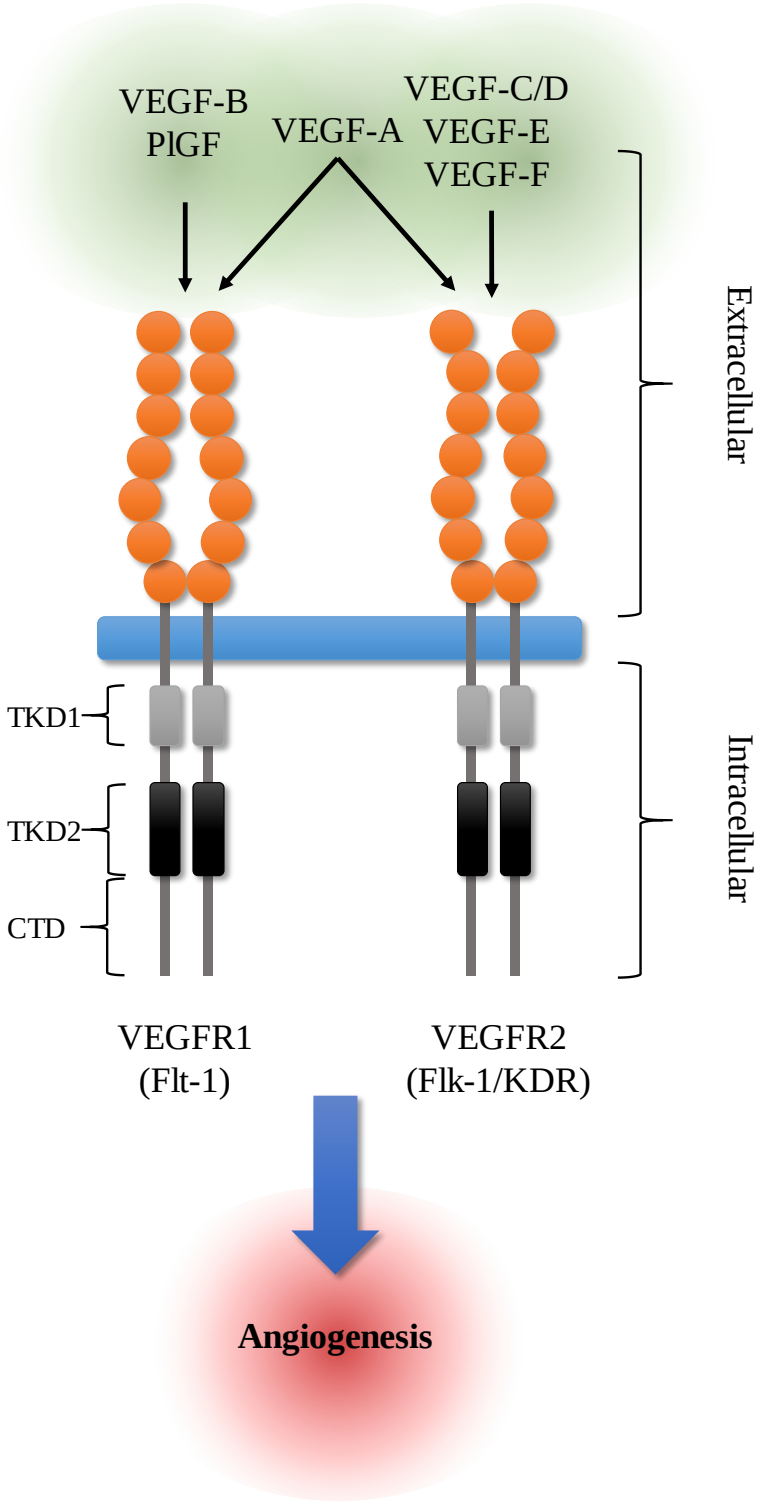


Figure 1-7. Vascular endothelial growth factor receptors (VEGFR) 1 and 2 and ligand interactions

VEGF-A is the major ligand which mediates angiogenesis and signals through both VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR). The VEGFR1 can also be stimulated with VEGF-B and Placental growth factor (PlGF), while VEGFR2 is a receptor for the ligands proteolytically processed VEGFC/D, VEGF-E, and VEGF-F. **CTD**, C-terminal domain; **TKD1**, ATP-binding domain, **TKD2**, phosphotransferase domain.

1.4.2.3 ***Regulation of angiogenesis by platelets (in vitro and in vivo)***

In very early studies, Knighton and colleagues demonstrated that thrombin-activated pellets of platelets could promote angiogenesis in an *in vivo* corneal neovascularization model (Knighton et al. 1982). Later, in a seminal study by Pipili-Synetos and colleagues, platelets were shown to promote capillary-like structure formation by human umbilical vein endothelial cell (HUVEC) *in vitro* via a direct interaction in the Matrigel tube formation assay (Pipili-Synetos, Papadimitriou & Maragoudakis 1998). Others demonstrated that platelets can induce HUVEC proliferation *in vitro* and that platelet adherence to VEGF-treated ECs was significantly inhibited with blocking antibodies against fibrinogen and GPIIb/IIIa, or by blocking TF activity or thrombin (Verheul et al. 2000b). While the contents released from thrombin-activated platelets could not promote HUVEC tube formation in the study by Pipili-Synetos and colleagues (Pipili-Synetos, Papadimitriou & Maragoudakis 1998), more recent studies have shown the opposite (Etulain et al. 2013; Kandler et al. 2004). The released contents from thrombin-activated platelets induced increased matrix-metalloprotease (MMP)2 release, migration, proliferation and tube formation by HUVECs (Kandler et al. 2004). In addition, tube formation mediated by platelet-released contents was shown to be independent of the VEGF content from platelets and could be completely inhibited with aspirin, an inhibitor of platelet cyclooxygenase-1 (COX-1) with irreversible effects on platelet function (Etulain et al. 2013). This result suggested that the platelet pro-angiogenic effects may be a result of several pro-angiogenic factors released from platelets, and further highlight aspirin as a candidate drug for therapeutically targeting angiogenesis.

In further support via the *in vitro* rat aortic ring model, Brill and colleagues demonstrated that platelets induce vessel growth dose-dependently, and that both platelets and platelet-released contents promote angiogenesis, although a higher dose was required for similar effects using platelet released contents (Brill, Elinav & Varon 2004). In this model, platelet-induced angiogenesis was completely inhibited following combinatorial antibody blocking of VEGF, bFGF, and PDGF.

The mechanism whereby platelets regulate angiogenesis is not fully understood since they contain both pro- and anti-angiogenic growth factors. Although, as previously mentioned in section 1.3, it is now recognised that platelets contain distinct sub-populations of α-granules with separate pro- and anti-angiogenic factors which are differentially released based on the stimulus (Chatterjee et al. 2011; Italiano et al. 2008; Ma et al. 2005). Through this regulated process, platelet-released contents can either promote or inhibit the migration and formation of capillary like structures by HUVECs *in vitro* (Battinelli, Markens & Italiano 2011).

Several *in vivo* studies have also shed light on the modulatory role of platelets in angiogenesis. Using the cornea micropocket assay, Kisucka and colleagues showed that platelets promote the formation of angiogenic vessels and prevent haemorrhage of newly formed vessels (Kisucka et al. 2006). In addition, they also established that angiogenesis was not affected by the absence of dense or lysosomal granules (Kisucka et al. 2006). While no model was available to study the involvement of α-granules in the aforementioned study, it is likely that α-granules are the primary regulators of the process since they contain an abundance of biological factors which regulate both physiological and pathological angiogenesis (Peterson et al. 2010).

1.5 **Angiogenesis in malignancy**

Angiogenesis can occur in a number of pathological conditions including tumour growth, rheumatoid arthritis, and retinopathies (Folkman 1995). Judah Folkman was the first to recognise in 1971 that without a blood supply, tumours can only grow to 1-2mm^3 (Folkman 1971). The acquired capacity for a tumour to induce angiogenesis is now recognised as a hallmark feature of cancer (Hanahan & Weinberg 2011). The uncontrolled cell proliferation and increased metabolic activity in tumours results in an hypoxic environment with increasing requirements for oxygen and nutrients in order to sustain growth (Bertout, Patel & Simon 2008). This, in turn, activates angiogenesis as a mechanism to obtain further delivery of oxygen and nutrients and removal of waste from the tumour microenvironment, which permits tumour growth beyond 1-2mm^3. Of significant concern in cancer is that once tumours acquire a blood supply this ultimately leads to expansive growth and also the capacity for vascular supported metastasis (Hillen & Griffioen 2007).

As in physiological angiogenesis, VEGF is a major regulator of tumour angiogenesis and this was demonstrated with antibody inhibition of VEGF or its receptor, VEGFR2, resulting in restricted tumour growth and invasion (Kim et al. 1993; Skobe et al. 1997). In addition, cancer cells transfected to express VEGF caused elevated tumour angiogenesis, tumour growth and metastasis *in vivo* (Kondo et al. 2000). Elevated levels of VEGF are a common finding in the serum or plasma of cancer patients and correlate with poor prognosis (Kut, Mac Gabhann & Popel 2007). Whilst it is known that cancer cells themselves can release VEGF, studies showed that VEGF levels are also reflective of elevated platelet counts, suggesting that platelets, as providers of VEGF, may also mediate angiogenesis and tumour growth (Pinedo et al. 1998; Verheul et al. 1997)

1.6 **Platelets in malignancy**

Abnormalities in the coagulation system of cancer patients were first recognised by Armand Trousseau in 1865 (Trousseau 1865). Then in 1872, Riess reported on thrombocytosis (defined as a platelet count of >450,000/mm^3) in cancer patients with solid tumours (Riess 1872). Thrombocytosis and high turnover of platelet counts have since become a common finding in patients with cancer (Sun et al. 1979). In more recent reports, thrombocytosis has been associated with poor prognosis in several cancers including early and metastatic breast cancer (Stravodimou & Voutsadakis 2013; Taucher et al. 2003), lung (Pedersen & Milman 1996) and ovarian cancer (Stone et al. 2012). Furthermore, a meta-analysis by Rothwell and colleagues demonstrated that daily aspirin use is associated with a reduction in long term risk (Rothwell et al. 2010) as well as short term incidence (Rothwell et al. 2012a) and mortality due to cancer (Rothwell et al. 2010).

The potential involvement of platelets in tumour angiogenesis was based on evidence that angiogenesis under normal conditions is regulated by the pro and anti-angiogenic factors released by platelets (Folkman 1971; Pinedo et al. 1998). Taken together, abundant evidence exists to support that platelets are regulators of tumour angiogenesis and that interactions between platelets and cancer cells contribute to metastasis development (Gay & Felding-Habermann 2011; Walsh, Metharom & Berndt 2015).

1.6.1 **Platelets in tumour angiogenesis and vessel stabilisation**

As previously discussed in section 1.4, platelets in the circulation can be activated following contact with damaged endothelium, responding to restore and repair the vasculature by releasing a myriad of factors from their granules. Studies led by Folkman proposed that in the tumour vasculature platelets can adhere and become activated through various means including, (1) reduced blood flow from the elevated interstitial pressure, (2) exposure to sub-endothelial matrix proteins due to the leaky nature of tumour vessels, and (3) exposure to vWF expressed on ECs activated by proangiogenic stimuli in the tumour vasculature (Pinedo et al. 1998). Activated platelets can secrete a wide range of growth factors that act to promote angiogenesis, for example; VEGF, bFGF, PDGF, EGF, but also to inhibit angiogenesis for example; TSP-1, angiostatin, endostatin, and PF4 (see Table 1-1). Taken together, platelets possess the ability to promote and/or inhibit angiogenesis.

Regarding a pro-angiogenic role for platelets in cancer, an association between platelets and tumour vascularisation is recognised, although this has not been directly shown. Verheul and colleagues demonstrated that activated platelets could be identified in tumour specimens of patients with soft tissue sarcomas, along with dense vascularisation and elevated levels of VEGF in the intratumoural fluid (Verheul et al. 2000a). This work supported the idea that platelets are readily activated in malignancy and contribute to tumour angiogenesis. Other studies have found that platelets isolated from lung cancer patients had >50% expression of P-selectin (a marker of platelet activation) while those isolated from healthy donors showed <10% P-selectin expression (a non-activated resting state) (Gong et al. 2012a). Furthermore, Qi and colleagues identified in human biopsies that platelets are in close association with cancer cells of breast cancer, colorectal cancer, gastric cancer, and hepatocellular cancer (Qi et al. 2015). In addition, mouse models have

shown that platelets promote tumour growth and angiogenesis in various tumour models including breast cancer (Kuznetsov et al. 2012), melanoma (Trikha et al. 2002), pancreatic cancer (Ringvall et al. 2011), colorectal cancer (Qi et al. 2015), and ovarian cancer (Stone et al. 2012; Yuan & Liu 2015). In the study by Trikha and colleagues, combined blockade of host integrins (platelet GPIIb/IIIa and endothelial $\alpha_v\beta_3$) and melanoma cell-expressed $\alpha_v\beta_3$ inhibited xenograft tumour formation in nude rats (Trikha et al. 2002). The role of platelets in malignancy in models with a platelet dysfunction has also been investigated. It has been documented that aspirin has inhibitory effects on platelet function, suppressing the TXA_2 pathway (Dovizio et al. 2013b; Patrignani, Filabozzi & Patrono 1982), and that administration of low dose aspirin (<25mg/kg) had anti-cancer effects in mouse models of neuroblastoma (Carlson et al. 2013) and colorectal cancer (Guillem-Llobat et al. 2016; Lichtenberger et al. 2017; Reuter, Zhang & Miller 2002).

Studies have established that platelets are also important in supporting and stabilising normal (Kisucka et al. 2006), inflamed (Goerge et al. 2008) and tumour microvessels (Ho-Tin-Noe et al. 2008). The induction of thrombocytopaenia, with anti-GPIbα antibody, resulted in intratumoural haemorrhage in murine Lewis lung carcinoma and B16/F10 melanoma models. The importance of platelet secreted proteins was highlighted when the intratumoural haemorrhage was prevented by transfusion of inactivated donor platelets but not thrombin degranulated donor platelets (Ho-Tin-Noe et al. 2009). Ho-Tin-Noe and colleagues then demonstrated that platelets protected the tumour vasculature from damage caused by innate immune cells (neutrophils and macrophages) in the tumour stroma, which induce haemorrhage in the absence of platelets (Ho-Tin-Noe et al. 2009).

As previously mentioned, it is also evident that platelets can release anti-angiogenic factors. For example, TSP-1, which is the most abundant protein contained within platelet α-granules, has been shown *in vitro* (Chu, Ramakrishnan & Silverstein 2013; Dawson et al. 1997; Good et al. 1990) and *in vivo* (Kopp et al. 2006; Tolsma et al. 1993) to inhibit angiogenesis. To investigate the relevance of these findings in tumour angiogenesis, Zaslavsky and colleagues utilised a murine model of lung carcinoma (Lewis lung carcinoma) together with TSP-1 deficient mice (Zaslavsky et al. 2010). Increased tumour burden and vascularisation was observed in mice injected with platelets from donor TSP-$1^{-/-}$ mice compared with those from wild-type mice, suggesting a critical function of TSP-1 in platelet-induced angiogenesis.

Due to the dual role of platelets, it has been suggested that they are neither promoters nor inhibitors of tumour growth and angiogenesis but may be modulators of the processes which may depend on the balance of stimulatory or inhibitory signals within the tumour microenvironment (Chatterjee et al. 2011; Etulain et al. 2011; Etulain et al. 2012; Italiano et al. 2008; Pilatova, Zdrazilova-Dubska & Klement 2012).

1.6.2 **Platelets in tumour metastasis**

A relationship between thrombosis and cancer has been known since the 1800s when French clinician Armand Trousseau recognised that solid cancers can cause thrombus formation at distal locations, and that the occurrence of these distant thrombi serves as an indicator of cancer, even prior to diagnosis (Trousseau 1865). Early studies demonstrated that thrombocytopaenia attenuates metastasis in several murine tumour models including colon, lung, melanoma and breast (Gasic, Gasic & Stewart 1968; Karpatkin et al. 1988; Pearlstein, Ambrogio & Karpatkin 1984). The metastatic potential of cancer cells was shown to correlate with their ability to aggregate platelets, a process defined as tumour-

cell induced platelet aggregation (TCIPA) (Jurasz, Alonso-Escolano & Radomski 2004). There is now an extensive body of evidence which shows that platelets support tumour metastasis via this process (Borsig 2008; Erpenbeck & Schon 2010; Gay & Felding-Habermann 2011; Leblanc & Peyruchaud 2016).

As with other cells (see section 1.2.2 above), platelets can mediate interactions with cancer cells via several cell surface receptors (Figure 1-8). Platelet receptors GPIIb/IIIa, GPIb-IX-V and P-selectin as well as cancer cell-expressed integrin αvβ3 and PSGL-1 are known to be important in TCIPA, and a deficiency of the receptors involved in TCIPA resulted in attenuated haematogenous spread of cancer cells (Borsig et al. 2001; Erpenbeck et al. 2010; Goetz et al. 1996; Gong et al. 2012a; Kim et al. 1998b; Lonsdorf et al. 2012; Nierodzik et al. 1991; Qi et al. 2014; Weber et al. 2016). It is also documented that cancer cells can express GPIIb/IIIa and GPIbα (Alonso-Escolano et al. 2004; Honn et al. 1992; Oleksowicz et al. 1995), and a GPIb-IX-V-like complex (Suter et al. 2001), which are normally exclusively expressed on platelets and megakaryocytes. Furthermore, tumour cells can release ADP and they can also express TF which causes the production of thrombin and release of ADP from platelets resulting in further platelet activation (Alonso-Escolano et al. 2004; Battinelli, Markens & Italiano 2011; Mueller et al. 1992; Ueno et al. 2000).

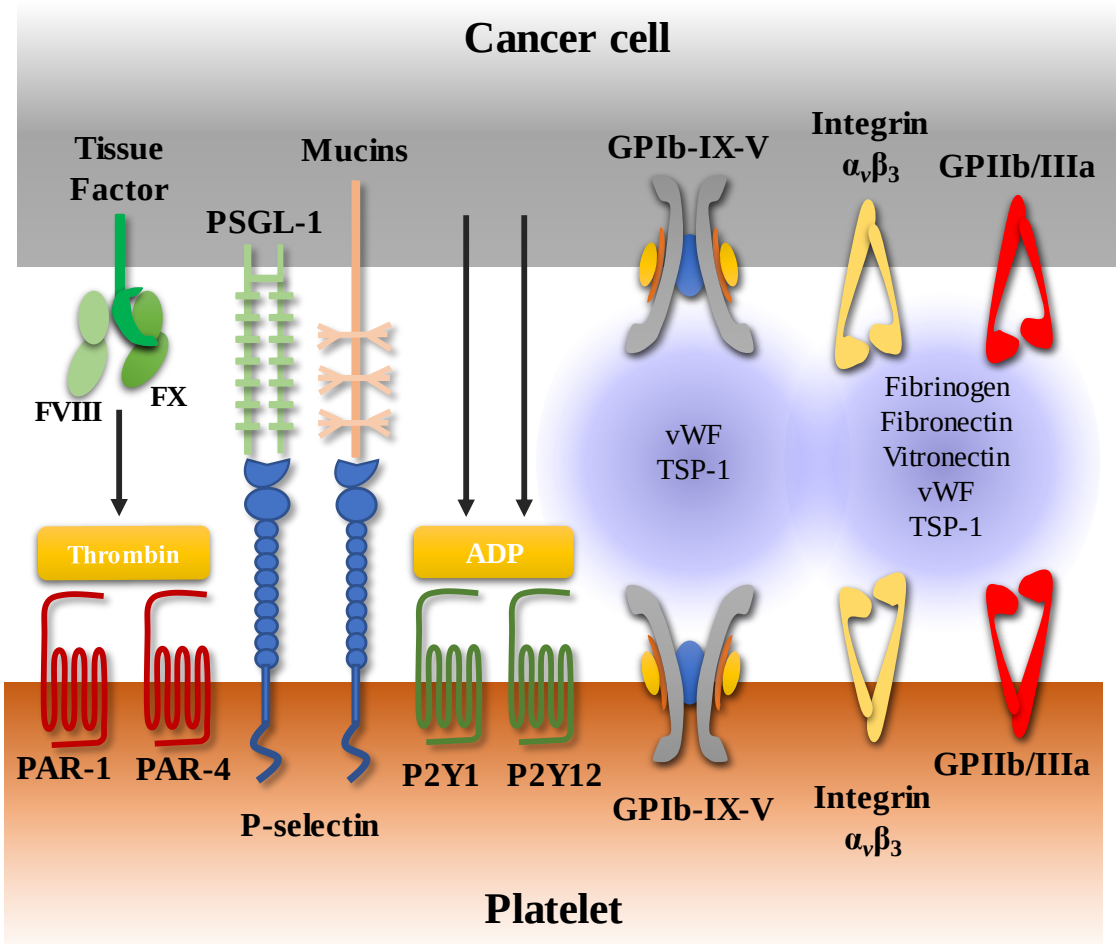


Figure 1-8. Cell surface receptor interactions between platelets and cancer cells

Interactions between platelets and cancer cells can occur through platelet P-selectin which binds to PSGL-1 or tethered mucins expressed on the cancer cell surface. GPIb-IX-V on cancer cells can also bind via RGD proteins to platelet-expressed GPIb-IX-V. RGD-containing proteins also mediate interactions through platelet integrin $\alpha_v\beta_3$ and GPIIb/IIIa with cancer cell expressed GPIIb/IIIa or aberrantly expressed integrin $\alpha_v\beta_3$ on cancer cells. Finally, cancer cells can release thrombin (by expressing TF on their surface) and ADP which can activate platelets via thrombin receptors (PAR-1 and PAR-4) and purinergic receptors (P2Y1 and P2Y12) on platelets. **ADP**, adenosine diphosphate; **FVIII**, factor VIII; **FX**, factor X; **GP**, glycoprotein; **PAR**, protease activated receptor; **TF**, tissue factor; **TSP-1**, thrombospondin-1; **vWF**, von Willebrand factor.

By mediating TCIPA, cancer cells obtain several advantages which promote survival and assist metastasis (Erpenbeck & Schon 2010; Jurasz, Alonso-Escolano & Radomski 2004). Firstly, the adherence of platelets and formation of aggregates on the cancer cell surface is documented to assist the formation of new metastases by circulating cancer cells by shielding and protecting them from natural killer (NK) cell mediated lysis (Nieswandt et al. 1999). Using genetic models of mice lacking fibrinogen or a G-protein which is essential in platelet activation (Gαq), the protective effect of platelets from NK cells was inhibited (Palumbo et al. 2005). These models highlighted that platelet activation and fibrinogen are important for NK-cell mediated cancer cell lysis. In addition, platelets have been shown to protect circulating cancer cells by forming a coating and transferring platelet-derived normal MHC class I onto the surface of cancer cells which impairs recognition by NK cells and cell death (Placke et al. 2012).

Secondly, the pro-coagulant nature of the platelet-cancer cell aggregates enhances their arrest at new extravasation sites by developing into microthrombi which are entrapped into microvasculature or by interactions of the activated platelets with endothelium (Li 2016; Menter et al. 2014; Rickles & Falanga 2001). Finally, TCIPA results in platelet activation which leads to release of platelet factors which have the potential to promote tumour growth and angiogenesis (Grignani et al. 1984; Mohle et al. 1997; Nierodzik et al. 1991; Pacchiarini et al. 1991).

As illustrated in Figure 1-9, it has been documented that platelets themselves (or their released contents) can promote a metastatic phenotype of cancer cells by inducing their epithelial-to-mesenchymal transition (EMT) (Dovizio et al. 2013b; Guillem-Llobat et al. 2016; Labelle, Begum & Hynes 2011; Orellana et al. 2015). Activation of EMT confers on cancer cells an elevated ability to migrate, invade and resist apoptotic signals (Hanahan & Weinberg 2011; Polyak & Weinberg 2009). Studies by Labelle and colleague showed

that platelet-derived TGF-β activates the Smad and NF-κB signalling pathways (phosphorylation of Smad2 and Smad-binding element-dependent transcription) which induces EMT and elevates lung metastasis in murine models of colon or breast carcinoma (Labelle, Begum & Hynes 2011). The aforementioned study demonstrated that direct contact between platelets and cancer cells, together with secreted TGF-β1, elevated the expression of a pro-metastatic gene profile.

In addition to promoting the metastatic process by EMT induction, platelets are also important in promoting metastatic progression (Labelle, Begum & Hynes 2014). Studies by Labelle and colleagues demonstrated that platelets guide the formation of the early metastatic niche (EMN), a specialised environment essential for the progression of metastasis (Labelle, Begum & Hynes 2014). They showed that upon contact with cancer cells, platelets secrete chemokines CXCL5 and CXCL7 which leads to the recruitment of granulocytes ($CD11b^{+}MMP9^{+}Ly6G^{+}$) to platelet-cancer cell aggregates, promoting the formation of the EMN. Furthermore, platelet depletion or impaired platelet aggregation (integrin β3-null murine model) also inhibited granulocyte recruitment to the EMN (Labelle, Begum & Hynes 2014), which are documented to promote metastasis by assisting cancer cell arrest and extravasation (Huh et al. 2010; Slattery & Dong 2003; Spicer et al. 2012). Labelle and colleagues further demonstrated that depletion of either granulocytes or platelets attenuated metastasis formation, although the effect was more potent with platelet depletion (Labelle, Begum & Hynes 2014). This highlights the importance of platelets in metastatic progression of cancers.

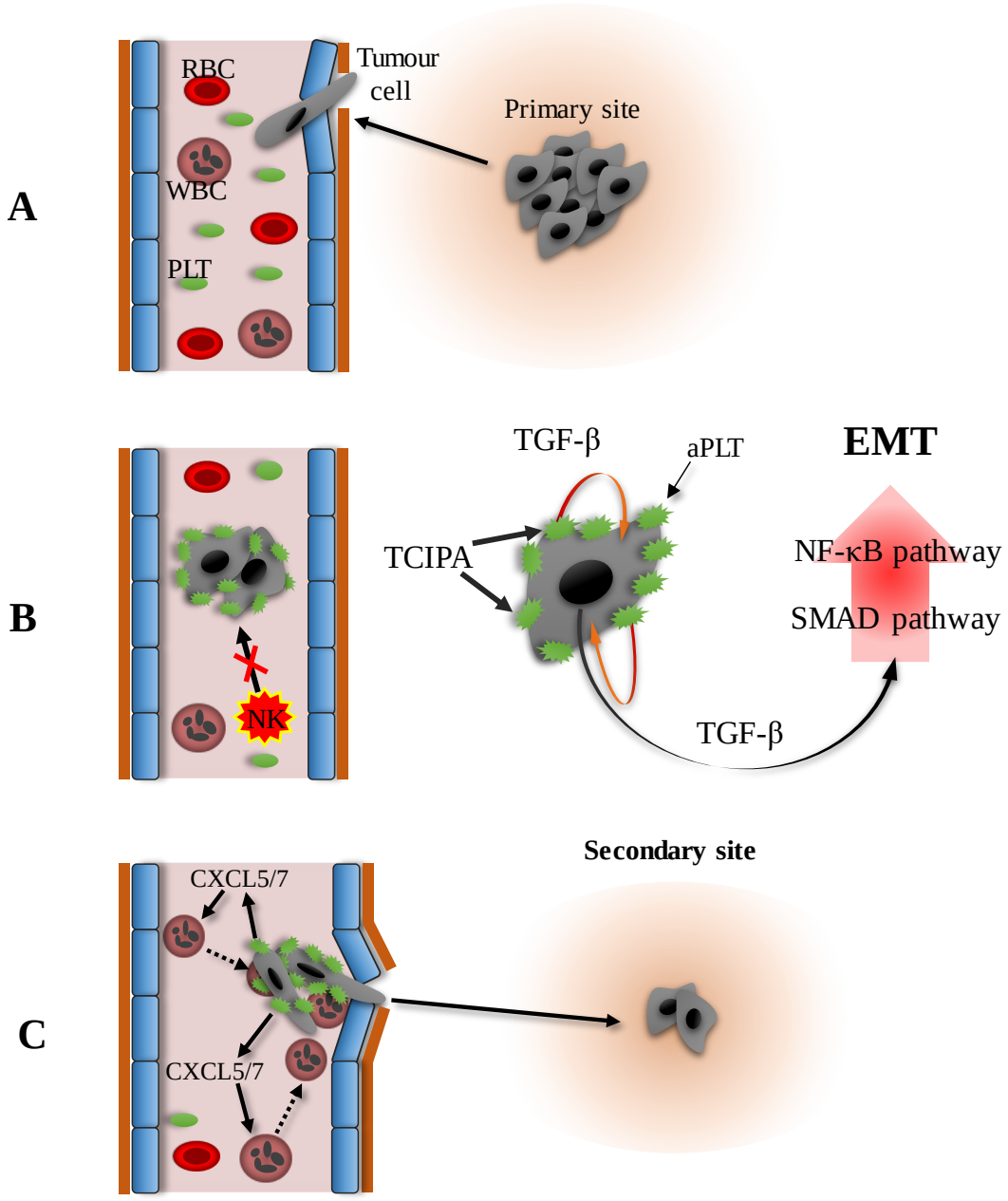


Figure 1-9. Epithelial-to-mesenchymal transition (EMT) induction by platelets and metastasis progression

(**A**) Tumour cells enter the circulation from the primary tumour site, coming into contact with cells circulating in the blood. (**B**) Upon contact with platelets, cancer cell-platelet aggregates form (TCIPA). Platelets form a protective shield to protect cancer cells from NK cell mediated cell death. The formation of aggregates results in platelet activation and platelet TGF-β release which activates EMT in cancer cells by activating the NF-κB and the SMAD pathways. (**C**) The activated platelets also release CXCL5 and CXCL7, leading to the recruitment of granulocytes to the metastatic niche which promote metastatic progression of cancer cells. **aPLT**, activated platelet; **CXCL**, C-X-C Motif chemokine ligand; **EMT**, epithelial-to-mesenchymal transition; **NK**, natural killer cell **PLT**, platelet; **RBC**, red blood cell, **TGF-β**, transforming growth factor-β; **WBC**, white blood cell.

1.7 **Tumour vascularisation**

As discussed previously, tumour vascularisation enables the delivery of oxygen and nutrients and the removal of waste products, allowing tumours to grow and also provides a pathway through which the cancer cells can enter the circulation and disseminate to distant sites (Carmeliet 2005; Folkman 1971, 1990). A major promoter of tumour vascularisation is hypoxia, occurring when there is an imbalance between oxygen availability and requirement in the tumour (Semenza 2000, 2007). Hypoxic conditions result in upregulation of hypoxia inducible factor-1 (HIF-1) which binds to hypoxia-response elements (HREs) to induce the activation of a number of genes which underpin tumour progression (Harris 2002; Semenza 2000; Vaupel 2004). This includes genes involved in the regulation of several solid tumour hallmarks, including vascularisation, maintenance of a stem cell phenotype, invasion and metastasis, overcoming apoptotic signals, genomic instability, immune response, metabolism, and drug resistance (Ke & Costa 2006; Wigerup, Pahlman & Bexell 2016).

In addition to driving sprouting angiogenesis, hypoxic conditions are documented to drive other tumour vascularisation pathways, including co-option of pre-existing vessels, intussusceptive microvascular growth, mosaic vessels lined by both tumour cells and endothelium, post-natal vasculogenesis, and vasculogenic mimicry (VM) (Hendrix et al. 2016; Hillen & Griffioen 2007; Kirschmann et al. 2012; van der Schaft et al. 2005). As discussed in more detail in the following section, VM is the formation of non-endothelial lined vascular-like structures which are formed by tumour cells via their capacity to secrete basement membrane proteins and are known to have plasma and blood carrying ability (Hendrix et al. 2003a; Maniotis et al. 1999). The formation of VM in tumours remains a novel area of research which is not fully understood (Hendrix et al. 2016; Seftor et al. 2012).

1.8 Vasculogenic mimicry in cancer

1.8.1 The discovery and clinical relevance of vasculogenic mimicry (VM)

The ability of highly aggressive tumour cells to form a patterned matrix of vascular-like structures with fluid-conducting capability was first described some years ago (Maniotis et al. 1999). Hendrix and colleagues observed that while VM structures stain positive for periodic acid-Schiff (PAS; highlights basement membranes) they were distinct from angiogenesis as they were negative for CD31, which although is not EC specific it is a common marker for ECs. This indicated that they comprised a basement membrane lined by tumour cells (Figure 1-10). Ultrastructurally, VM comprises of sheets of ECM recognised to be rich in laminin, collagen IV and VI, and heparin sulfate proteoglycans (Folberg & Maniotis 2004). The major differences between VM structures and endothelial-lined vasculature were; i) structures formed by the aggressive tumour cells are patterned, looping and interconnected, whereas angiogenic structures were organised into clusters of un-patterned vessels; ii) structures formed from VM do not contain ECs; and iii) the architecture of VM-formed structures, the interconnected loops and vessel-like structures, is characteristic of vasculogenesis (the de novo formation of microvasculature) (Maniotis et al. 1999).

The presence of plasma and red blood cells within VM structures revealed an alternate perfusion pathway to tumour angiogenesis, as well as a mechanism for aggressive cancer cells to disseminate (Hendrix et al. 2003a; Maniotis et al. 1999). Several studies have since demonstrated that VM structures can access the blood supply through forming a physical connection with endothelial-lined vessels or obtaining fluid through leaky endothelial-lined vessels (Folberg & Maniotis 2004; Frenkel et al. 2008; Ruf et al. 2003; Shirakawa et al. 2002a; Shirakawa et al. 2003).

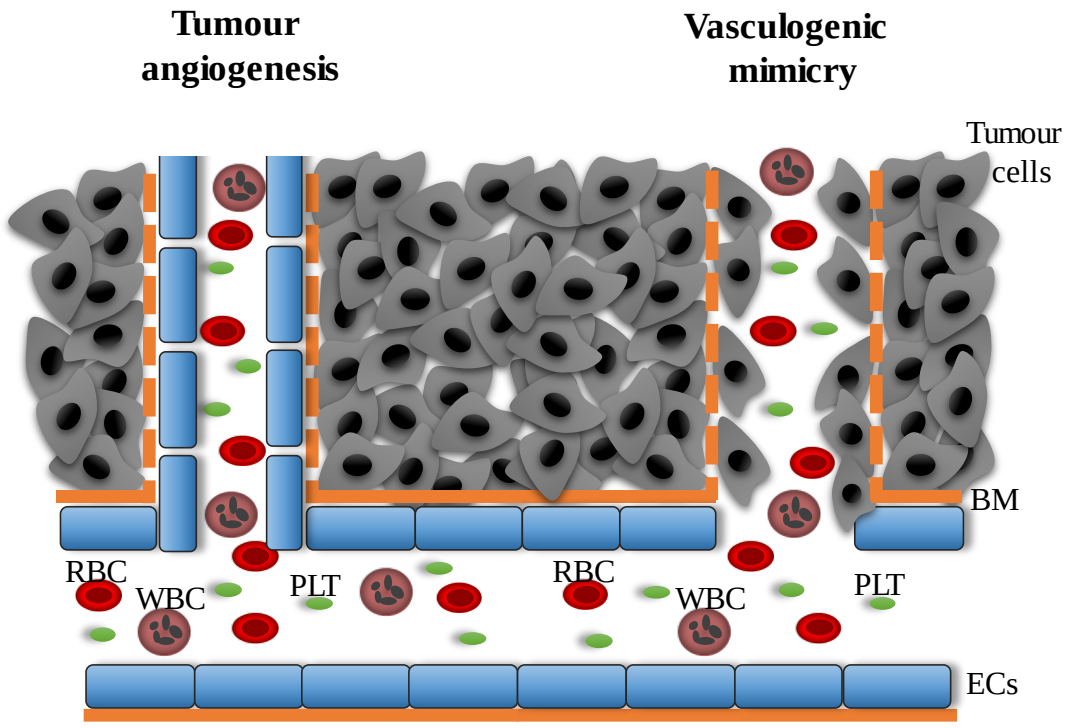


Figure 1-10. Features of tumour angiogenesis and vasculogenic mimicry

Tumour angiogenic structures (left) are leaky, vascular-like structures that are lined by endothelial cells (**ECs**) on a basement membrane (**BM**). In contrast, VM structures are leaky, vascular-like structures which are formed by aggressive tumour cells which secrete their own basement membrane. Both tumour angiogenic and VM structures have the ability to carry blood towards the tumour. **PLT**, platelet; **RBC**, red blood cell; **WBC**, white blood cell.

While VM was originally described in uveal and cutaneous melanoma (Maniotis et al. 1999; Seftor et al. 2002a), it has also been reported to occur in a number of tumours including breast cancer (Buijs et al. 2004; Plantamura et al. 2014; Shirakawa et al. 2001; Zhang et al. 2014a), ovarian cancer (Gao et al. 2009; Sood et al. 2002; Sun et al. 2014), prostate cancer (Sharma et al. 2002), colorectal cancer (Baeten et al. 2009; Liu et al. 2012) glioblastoma (Francescone et al. 2012; Scully et al. 2012) and malignant mesothelioma (Pulford et al. 2016). It is documented that VM vessels comprised an average of 40% of the total number of blood vessels in tumour specimens from melanoma patients (Itzhaki et al. 2013), an average of 15.5% in lymph node specimens from small cell lung cancer patients (Williamson et al. 2016), and 5-14% in human ovarian carcinoma (Sood et al. 2002). In a recent meta-analysis study by Yang and colleagues, the presence of VM was strongly associated with cancer differentiation, lymph node metastasis, distant metastasis, and tumour, node and metastasis (TNM) stage as well as with a significant reduction in overall survival in 11 different human cancers (Yang et al. 2016).

It has been demonstrated that highly invasive melanoma cells could form VM structures, while poorly invasive melanoma cells tended not to (Maniotis et al. 1999). To understand the mechanisms underpinning VM, differences in the molecular phenotype between highly aggressive and poorly aggressive melanoma have been investigated. (Hess et al. 2001; Maniotis et al. 1999; Seftor et al. 2002a). Molecular studies identified VM signature genes involved in angiogenesis and vasculogenesis that were upregulated in aggressive cancer cells, including vascular endothelial (VE)-cadherin (*CDH5*), erythropoietin-producing hepatocellular receptor A2 (*EPHA2*), and laminin 5 γ2-chain (*LAMC2*) (Hendrix et al. 2001; Hess et al. 2001; Seftor et al. 2001).

1.8.2 **Signalling pathways in VM**

Several signalling pathways are known to be involved in VM formation, including those seen in vasculogenesis (VE-cadherin, EphA2, and MMPs), embryonic vasculogenesis (Notch4-Nodal and Wingless), and hypoxia-related (HIF-1/HRE binding) processes (Hendrix et al. 2016; Kirschmann et al. 2012). Genes essential for aggressive tumours to form VM structures include; VE-cadherin, MMPs (membrane type-1-matrix metalloproteinase (e.g. MMP2, and MMP9), laminin 5γ2 chains, and EpherinA2 (EphA2) (Hendrix et al. 2001; Seftor et al. 2001), which are discussed in detail below. Other molecules also shown to be involved in VM, which will not be discussed in detail here, have been summarised in Table 1-3 and are further illustrated in Figure 1-11.

1.8.2.1 ***EPHA2***

EphA2 is a receptor tyrosine kinase that belongs to a family of 14 receptor tyrosine kinases (Pasquale 1997). Hess and colleagues found EphA2 to be involved in VM formation by highly aggressive melanoma cells, and protein expression is only detected in highly aggressive cells (Hess et al. 2001). Inhibition of EphA2 resulted in attenuation of tube formation in a 3D culture system. This early work has been supported by more recent studies revealing that in VM, EphA2 mediates signals through phosphatidylinositol-3-kinase (PI3K) (Chen et al. 2011; Hess et al. 2003; Liang et al. 2014; Lu et al. 2013).

1.8.2.2 *VE-cadherin*

Like EphA2, protein levels of VE-cadherin (CD144) were also found to be upregulated on highly aggressive melanoma cells (Hendrix et al. 2003a). VE-cadherin is an endothelial-specific cell surface adhesion molecule and has been shown to play a role in VM formation by highly aggressive cancer cells (Hendrix et al. 2001). In melanoma, VE-cadherin was shown to be co-localised with EphA2 at cell-cell adhesion junctions, and knockdown of VE-cadherin resulted in reorganisation of EphA2 away from the cell-cell junctions. Interestingly, knockdown of EphA2 did not affect the localisation of VE-cadherin away from the cell-cell junctions (Hess et al. 2006). The interaction of VE-cadherin with EphA2 can activate the phosphoinositide 3-kinase (PI3K) pathway leading to MMP14 and MMP2 activation which are able to cleave laminin γ2 chain into promigratory fragments that are essential to VM formation (Hess et al. 2003; Seftor et al. 2001).

1.8.2.3 *LAMC2*

Laminin-5 is an ECM protein consisting of α_3, β_3, and γ2 subunits which are encoded by LAMA3, LAMB3, and LAMC2 genes, respectively (Gerecke et al. 1994; Kallunki et al. 1992; Ryan et al. 1994). Microarray analysis demonstrated that laminin-5 γ2 chain is significantly elevated in cells capable of VM (Seftor et al. 2001). It has been demonstrated that MMP2 can cleave laminin-5 γ2 chain into γ2' and γ2x fragments, which promote migration of human mammary epithelial cell and the non-tumorigenic breast epithelial cell line, MCF10. This effect could be blocked by a MMP2 inhibitor (Giannelli et al. 1997). A similar effect was observed with a panel of cancer cell lines, revealing that MMP14 directly cleaves the laminin γ2 chain in a similar manner to MMP2, and promotes cancer cell migration (Koshikawa et al. 2000). Seftor and colleagues demonstrated that

poorly aggressive melanoma cells which were only able to form VM structures on gel matrix preconditioned with highly aggressive cells, lost their VM capacity when grown on a pre-conditioned gel matrix with neutralising antibodies against laminin γ2 chain antibodies (Seftor et al. 2001). Cleavage of laminin γ2 chain into promigratory γ2' and γ2x fragments has been demonstrated to be essential for VM formation (Hess et al. 2003; Seftor et al. 2001).

1.8.2.4 ***MMPs***

Matrix metalloproteases (MMPs), are a large family of proteases which are responsible for tissue remodelling via their degradation of ECM proteins (reviewed in Page-McCaw, Ewald & Werb 2007). MMPs are known to be over-expressed in highly malignant cancer cells (able to form VM) in comparison to poorly invasive cancer cells (Maniotis et al. 1999). MMPs that play a role in VM by cancer cells include MMP2, MMP14, MMP1 and MMP9 (Sood et al. 2004). Seftor and colleagues demonstrated through immunostaining that MMP1, 2, 9 and 14, co-localise with VM patterned networks formed by aggressive melanoma cells on collagen I gels (Seftor et al. 2001). The expression of MMP2 and MMP9 proteins has also been associated with VM in human pancreatic cancer (Guo et al. 2014). Cleavage of laminin γ2 chain, an important determining factor in VM, can occur through MMP14 and MMP2 activity (as discussed above). Interestingly, although MMP9 is also associated with VM formation in pancreatic cancer (Guo et al. 2014), contradictory findings have demonstrated that it is unable to cleave laminin γ2 chain (Giannelli et al. 1997) and inhibition of its activity does not alter VM forming ability by highly malignant cancer cells (Seftor et al. 2001).

Hess and colleagues investigated the regulation of MMPs in VM and demonstrated that phosphoinositide 3-kinase (PI3K), which signals through Akt, is necessary in VM formation (Hess et al. 2003). Blocking PI3K reduced expression of active MMP2 and MMP14, caused a reduction in released laminin γ2 chain fragments, and abolished the ability of highly aggressive melanoma cells to form VM structures on collagen I matrix (Hess et al. 2003). Furthermore, VE-cadherin/EphA2 signalling can activate PI3K, in turn activating MMP activity and resulting in pro-migratory signals via the cleavage of laminin γ2 chain. The identification of several molecules involved in VM has provided several potential therapeutic strategies to target VM.

Table 1-3. Factors and mechanisms involved in VM

Factor/ mechanism	Description of function	Reference
cAMP	• Increased levels of cAMP inhibit VM by activating Epac/Rap1 pathway, & by inhibiting ERK & PI3K/Akt signalling	(Lissitzky et al. 2009)
COX-2	• COX-2 inhibition impairs VM by blocking PGE_2 formation	(Basu et al. 2006; Liu et al. 2011a)
EMT, ZEB1/2 and Twist1	• VM is inhibited by downregulation of Flk-1 when ZEB1 is knocked down. • ZEB2 overexpression promotes TGF-β-induced EMT (by ↑ levels of VE-cad, VEGFR1/2, MMP2 & MMP9) • Twist-1 downregulates E-cad & activates MMP2 & MMP9	(Li et al. 2017; Liu et al. 2012; Sun et al. 2010; Yang et al. 2015; Zhang et al. 2014a; Zhao et al. 2011)
Desmoglein 2	• Inhibition or knockdown of DSG2 ↓VM by impairing cell-cell interactions	(Tan et al. 2016)
EphA2/ VE-cadherin	• VE-cad/EphA2 interaction leads to PI3k pathway activation, ↑MMPs (2 & 14) activation & cleavage of Ln-5 γ2.	(Hendrix et al. 2001; Hess et al. 2006; Lu et al. 2013; Margaryan et al. 2009)
FAK	• Overexpressed EphA2 promotes FAK-dependent MMP2 expression. • pFAK leads to pERK1/2, which ↑MMPs (2 & 14) activity	(Duxbury et al. 2004; Hess & Hendrix 2006; Hess et al. 2005)
Galectin-3	• Gal-3 silencing leads to EGR-1 overexpression & ↓VM (by inhibiting VE-cad, IL-8, & MMP2)	(Mourad-Zeidan et al. 2008)
Hypoxia, HIF-1α	• HIF-1α promotes VM by up-regulating neuropilin-1 • Knockdown of HIF-1α ↓ EphA2, VE-cadherin & laminin-5γ2 expression	(Misra, Bajaj & Kale 2012; Sun et al. 2007; Tang et al. 2014; Zhao et al. 2012)
MMPs, Ln-5 γ2 chain	• MMPs cleave Ln-5 γ2 chain, inducing a more invasive phenotype.	(Seftor et al. 2002c; Seftor et al. 2001)

Nodal/Notch	• Activating Nodal ↑ VM by increasing VE-cad expression. • Nodal ↑VM through Smad2/3 pathway by upregulating Slug, Snail and c-Myc to induce EMT	(Gong et al. 2016; Hardy et al. 2010; McAllister et al. 2010; Topczewska et al. 2006)
PI3K /Akt	• PI3k/Akt activation ↑ MMP activation (2 & 9) resulting in cleaved Ln-5 γ2 chain	(Hess et al. 2003; Lissitzky et al. 2009; Lu et al. 2013)
TF and TFPI1/2	• TFPI1 assists perfusion in VM • TFPI2 inhibition cause ↓ VM	(Ruf et al. 2003)
VEGF, VEGFR1/2	• VEGF ↑ VM-related genes (VE-cad, EphA2, MMP2 and MMP9). • VEGFR1 inhibition ↓ melanoma VM • VEGFR2 knockdown ↓VM by glioma stem-like cells.	(Frank et al. 2011; Mei et al. 2008; Vartanian et al. 2011a; Wang et al. 2008; Yao et al. 2013)

cAMP, cyclic adenosine monophosphate; **COX-2**, cyclooxygenase-2; E-cad, epithelial cadherin, **EMT**, epithelial-to-mesenchymal transition; **EphA2**, erythropoietin-producing hepatocellular carcinoma; **FAK**, Focal adhesion kinase; **HIF-1α**, hypoxia inducible factor-1α; **IL-8**, interleukin-8; **PGE_2**, prostaglandin E_2; **Ln 5 γ2**, Laminin 5 gamma 2 chain; **MMPs**, matrix metalloproteases; **PI3K/Akt**, phosphoinositide 3-kinase/protein kinase B; **TF**, tissue factor; **TFPI**, tissue factor pathway inhibitor; **VE-cad**, vascular endothelial cadherin; **VEGF**, vascular endothelial growth factor; **VEGFR**, VEGF receptor; **VM**, vasculogenic mimicry; **ZEB**, Zinc finger E-box binding homeobox.

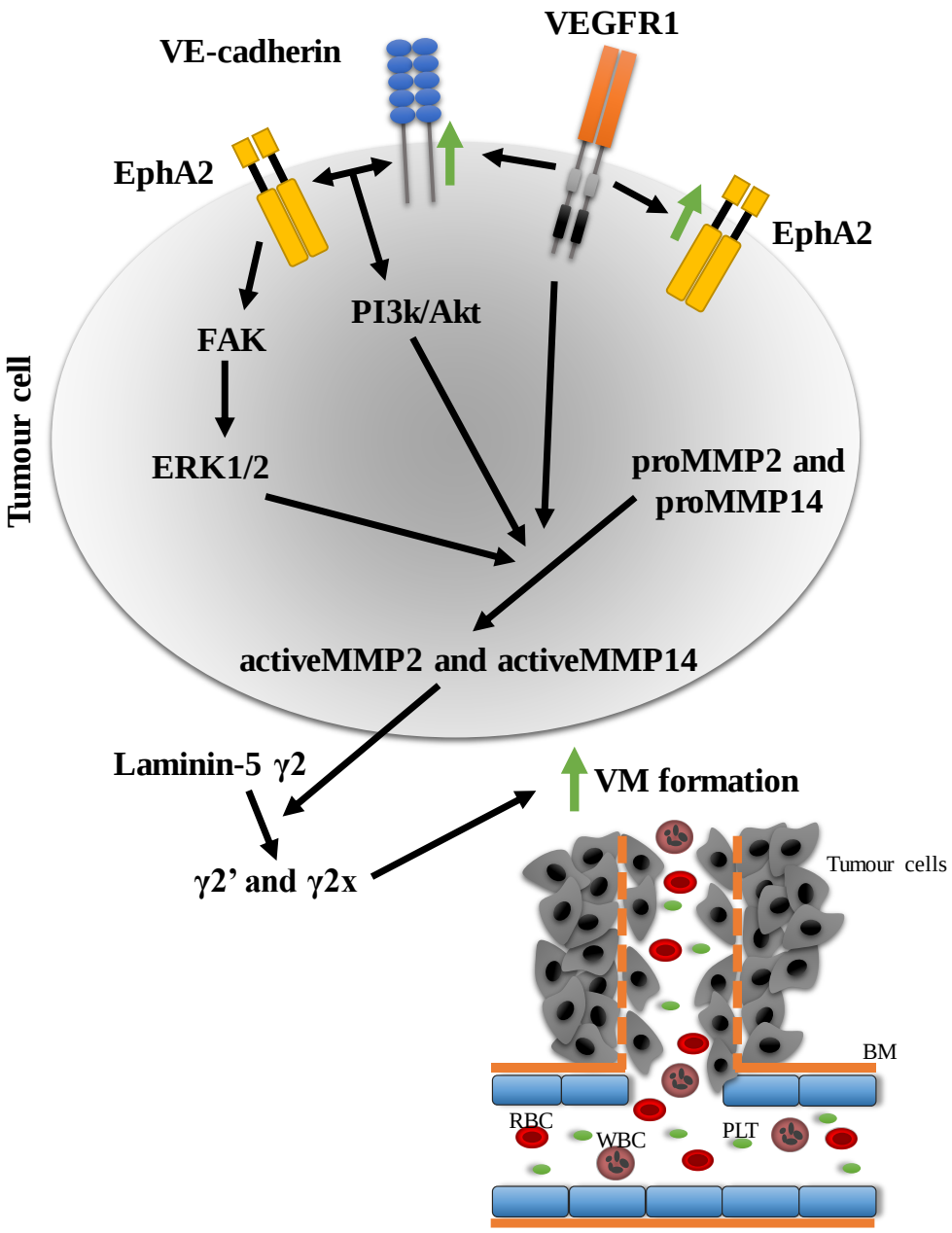


Figure 1-11. The vascular signalling pathway in vasculogenic mimicry (VM)

Genes involved in vascular signalling including VE-cadherin, EphA2, MMPs, and laminin-5 γ2 chain are essential for VM formation by tumour cells. VEGFR1 can upregulate both VE-cadherin and EphA2 expression, and MMPs. EphA2 alone can induce FAK-dependent ERK1/2 activation and MMP activation. VE-cadherin and EphA2 can co-localise at the cell surface to activate the PI3k/Akt signalling pathway and MMP activation. The activation of MMP2 and MMP14 which can both cleave laminin-5 γ2 chain into promigratory γ2' and γ2x is essential for VM formation by tumour cells. **BM**, basement membrane; **EphA2**, ephrin type-A receptor 2; **ERK**, extracellular-signal-regulated kinase; **FAK**, focal adhesion kinase; **MMP**, matrix metalloproteinase; **PI3k/Akt**, phosphatidylinositol 3-kinase/Protein Kinase B; **PLT**, platelet; **RBC**, red blood cell, **VE**-cadherin, vascular endothelial cadherin; **VEGFR1**, vascular endothelial growth factor receptor 1; **WBC**, white blood cell.

1.8.3 **Therapeutic strategies to target VM**

CVM-1118 (TRX 818) is the first and only anti-VM drug developed and is now in Phase I clinical trials in the US (NCT02507544) and Taiwan (NCT02703298) studied in patients with advanced cancer. In melanoma cells *in vitro*, the drug causes a dose dependent inhibition of VM formation and tube length, a reduction in proliferation, reduced viability and increases apoptosis (Hendrix et al. 2016). The aforementioned work also demonstrated that CVM-1118 reduced the expression of Nodal/Notch pathway (and downstream regulator pSMAD2) as well as VEGF-A at the mRNA level.

Although clinical trials aimed at testing VM-inhibiting drugs currently are scarce, there is experimental evidence highlighting other potential drugs that may be used to target VM. For example, thalidomide, an immunomodulating drug with anti-angiogenic properties, inhibits VM in mice with B16F10 tumours by reducing mRNA expression of vasculogenic factors including MMP2, and MMP9 (Zhang et al. 2008). Zoledronic acid, a bisphosphonate drug used to treat high blood calcium levels, also has anti-VM effects in osteosarcoma cells *in vitro* (Fu et al. 2011). In highly aggressive melanoma, nicotinamide has shown promising effects as a potential VM inhibiting drug (Itzhaki et al. 2013). Curcumin, a chemical compound derived from the root of the rhizome *Curcuma longa,* is demonstrated to have inhibitory effects on VM in melanoma and glioblastoma (Chen et al. 2011; Yadav & Aggarwal 2011), hepatocellular carcinoma (Chiablaem et al. 2014; Liang et al. 2014), and squamous cell carcinoma (Hu et al. 2015). These effects are medicated through targeting the signalling pathways EphA2/PI3K/MMP, STAT3 with PI3K/AKT and JAK-2/STAT-3, respectively. Norcantharidin, a synthetic anti-cancer compound derivative of cantharidin from the blister beetle *Mylabris phalerata* Pallas, also mediates inhibitory effects both *in vitro* and *in vivo* on VM in highly aggressive gallbladder cancer, resulting in abrogation of VM upon treatment through downregulation

of the PI3-K/MMPs/Laminin-5γ2 signalling pathway (Wang et al. 2014; Zhang et al. 2014b). Ginsenoside Rg3, a trace tetracyclic triterpenoid saponin which is extracted from the ginseng plant, has proved effective in inhibition of pancreatic cancer VM *in vitro* and *in vivo* through downregulation of VE-cadherin, EphA2, and MMPs (Guo et al. 2014). Finally, extensive investigations have also revealed that micro-RNAs have promising effects in the inhibition of VM by human glioma cells (Wu et al. 2011; Xue et al. 2016), and cervical cancer cells *in vitro* (Wan et al. 2014).

Whilst anti-angiogenic therapies show some inhibition of tumour growth *in vitro*, *in vivo* and clinically, results have been quite modest (Faivre et al. 2006; Hurwitz et al. 2004; Montero et al. 2012). Nonetheless, in efforts to find therapeutic strategies for inhibiting VM, studies have also tested the potential effectiveness of anti-angiogenic drugs. Anti-angiogenic drugs including bevacizumab (Avastin; VEGF inhibitor), Anginex (galectin-1-binding peptide), endostatin (Endostar), and TNP-470 (AGM-1470; TNF-α targeting drug) showed no effect on VM formation *in vitro* (Kirschmann et al. 2012; Seftor et al. 2012; van der Schaft et al. 2004). In fact, several studies have observed that anti-angiogenic treatments (bevacizumab, sunitinib, sorafenib, or anti-VEGFR2 blocking antibody), can promote VM as well as malignant progression by inducing hypoxia in tumours (Ebos et al. 2009; Paez-Ribes et al. 2009; Sun et al. 2017; Xu et al. 2012; Zhang et al. 2014a). These studies highlight the need to search for new therapeutic agents which target both angiogenesis and VM, in order to successfully inhibit tumour growth and support patient survival.

1.9 Platelets in vasculogenic mimicry

There are several supporting lines of evidence to suggest that platelets have the potential to regulate VM formation by aggressive tumour cells. Firstly, studies have shown that RBCs and plasma are detected in VM structures (Folberg & Maniotis 2004; Maniotis et al. 1999), indicating that platelets can enter VM structures and potentially interact with the tumour cells lining these structures. Secondly, elevated interstitial fluid pressure in tumours and stasis of blood can result in platelet interaction with tumour cells via several surface receptors such as GPIIb/IIIa and P-selectin expressed on activated platelets with integrin $\alpha_v\beta_3$ and PSGL-1, respectively (Jurasz, Alonso-Escolano & Radomski 2004; Pinedo et al. 1998). Thirdly, VM structures are known to contain collagen I, III and IV, as well as abundant amounts of laminin-5 (Folberg & Maniotis 2004), proteins with which platelets can interact via their expression of relevant surface receptors. These receptors include GPIIb/IIIa (mediating interactions with fibrinogen, fibrin, vWF, TSP-1, fibronectin and vitronectin), $\alpha_v\beta_3$ (binding to vitronectin, fibrinogen, vWF, TSP-1), $\alpha_2\beta_1$ (collagen binding), and integrin $\alpha_6\beta_1$ (laminin binding), and $\alpha_5\beta_1$ (fibronectin binding) (Kauskot & Hoylaerts 2012; Nieswandt, Varga-Szabo & Elvers 2009).

In further support, studies have shown that integrins which are important in tumour angiogenesis (including platelet GPIIb/IIIa, endothelial $\alpha_v\beta_3$, and tumour cell-expressed $\alpha_v\beta_3$) are also important for VM (Trikha et al. 2002; Vartanian et al. 2011b). Antibody-mediated inhibition of integrins $\alpha v\beta_3$ and $\alpha v\beta_5$ inhibited VM formation by aggressive melanoma cells *in vitro* (Vartanian et al. 2011b). In addition, platelets may bind to VM structures leading to platelet aggregation and activation and thus release of pro- or anti-angiogenic factors. Finally, platelets have been shown to induce a phenotypic switch in tumour cells in circulation towards an EMT phenotype (Dovizio et al. 2013b; Labelle,

Begum & Hynes 2011), which is shown to be a phenotype that is activated in VM formation (Liu et al. 2012; Sun et al. 2011; Yang et al. 2015). This suggests that platelets may promote VM formation and support tumour growth.

The central hypothesis of this thesis is that platelets promote VM formation by breast cancer and melanoma. Herein, human cancer cell lines of breast cancer and melanoma will be utilised to determine how platelets interact within the VM environment. Furthermore, a murine model of breast cancer, within which platelet function will be impaired, will then be used to elucidate the regulation of VM characteristics, and VM-specific markers in breast cancer *in vivo*. The specific aims of research in this thesis are:

1. To determine the role of platelets in VM formation by human breast cancer and melanoma cell lines *in vitro*
2. To investigate the mechanisms involved and compare the importance of direct interaction versus release of soluble factors
3. To investigate the *in vivo* role of platelets in VM formation by human breast cancer cell lines using a xenograft mouse model with aspirin-induced platelet inactivation.

The research aims to elucidate the role of platelets in VM structure formation, and provide further insight into the mechanism of VM. This research will significantly contribute to the field of cancer research by potentially identifying novel therapeutic targets against tumour vascularisation for the most aggressive cancers with the highest mortality, breast cancer and melanoma.

Chapter 2. Materials and Methods

2.1 Ethical statements

2.1.1 Human ethics for human umbilical cords and blood

The collection of primary human umbilical cords for EC isolation from veins was given ethical clearance from the Royal Adelaide Hospital Human Ethics Committee, Adelaide, SA, Australia (approval no. 050310). Informed consent was obtained from all subjects in accordance with the 'Declaration of Helsinki'.

2.1.2 Human ethics for the collection of peripheral blood

Ethics approval for the recruitment of participants and collection of peripheral blood was obtained from the University of South Australia's Human Research Ethics Committee (approval no. 0000031986). Informed written consent was obtained from all subjects.

2.1.3 Animal ethics

Animal experiments were approved by the Animal Ethics Committee of SA Pathology (approval no. 47-15) and conform to the guidelines established by the 'Australian Code of Practice for the Care and Use of Animals for Scientific Purposes'.

2.2 Materials

2.2.1 Animals

Female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, NOD.CB17-Prkdc^{scid}, at 6-8 weeks of age were obtained from the Animal Resources Centre (Canning Vale, Western Australia). On arrival, mice were housed in specific pathogen-free conditions in the SA Pathology Animal Care Facility (Adelaide, SA, Australia) at environmental (25°C) temperature and with a 12-hour light/dark cycle. Mice were fed standard rodent chow.

2.2.2 Human cancer cell lines

Human melanoma cell lines (CHL-1 and C32) and breast cancer cell lines (MDA-MB-231 and HS-578T) were from American Type Culture Collection (ATCC) and MDA-MB-231 LM2 cell line was kindly provided by Dr Joan Massagué (Sloan-Kettering Institute for Cancer Research, New York).

2.2.3 General reagents

Reagents were obtained from Sigma-Aldrich (St Louis, MO, USA), Invitrogen (Life Technologies), Chem Supply (Gilman, SA, Australia). Chemicals were analytical grade unless otherwise stated. Recipes for buffers and solutions are detailed in Appendix 1.

2.2.3.1 *Antibodies*

The antibodies used for flow cytometry and immunohistochemistry are detailed in Table 2-1.

2.2.3.2 *Primers*

Human specific PCR primers were designed using online Primer Blast (National Center for Biotechnology Information (NCBI)), and synthesised by GeneWorks (Hindmarsh, SA, Australia). Primer sequences are detailed in Table 2-2.

2.2.3.3 *Mammalian cell culture reagents*

A list of reagents used in tissue culture are detailed in Table 2-3.

2.2.3.4 *Molecular reagents*

Molecular reagents used are listed in Table 2-4.

2.2.3.5 *Miscellaneous reagents and consumables*

Other reagents and consumables used throughout this study are listed in Table 2-5.

Table 2-1. Antibody list

Name	Specificity (clone)	Antibody	Application & Dilution	Source (catalogue #)
IgG1-FITC	(MOPC-21)	Mouse monoclonal IgG1, κappa isotype Control	FC Isotype 1:5	BioLegend, San Diego, CA, USA (400110)
IgG1-PE	(MOPC-21)	Mouse monoclonal IgG1, isotype control	FC Isotype 1:5	BioLegend, San Diego, CA, USA (400114)
CD42b-FITC	Specifically binds to human CD42b (HIP1)	Monoclonal Mouse IgG1	FC 1:5	BD Biosciences, San Jose, CA (555472)
CD62P-PE	(AK4)	Monoclonal Mouse IgG1	FC 1:5	BioLegend, San Diego, CA, USA (304906)
Purified mouse IgG1	Raised against mouse myeloma protein (MOPC-21)	Monoclonal Mouse IgG1	FC Isotype 1:100	BD Biosciences, San Jose, CA, USA (555746)
CD162 (PSGL-1)	Raised against tyrosine sulfation consensus motif of PSGL-1 (KPL-1)	Monoclonal Mouse IgG1	FC 1° 1:100	BioLegend, San Diego, CA, USA (328802)
$\alpha_v\beta_3$	Recognises the CD51/CD61 complex (LM609)	Monoclonal Mouse IgG1	FC 1° 1:100	Millipore, Billerica, MA, USA (MAB1976)
Anti-mouse Dylight650	Raised against mouse IgG and light chains common to other mouse IgGs	Goat polyclonal IgG	FC 2° 1:100	Abcam, Cambridge, UK (ab96882)
CD36	(JC63.1)	Mouse IgA	FC 1° 1:2000	Cayman chemical, Ann Arbor, MI, USA (18150)
Purified Mouse IgA	(S107)	Monoclonal Mouse IgA	FC Isotype 1:2000	GeneTex, Irvine, CA, USA (GTX35045)
CD41	Raised against N terminal domain of Human CD41.	Rabbit IgG polyclonal	IHC 1° 1:1200	Abcam, Cambridge, UK (ab63983)
CD31	Raised against (residue 738) of human CD31. Also detects mouse CD31.	Rabbit IgG polyclonal	IHC 1° 1:250	Bethyl lab, Montgomery, TX, USA (IHC00055)
Goat-anti rabbit biotin	Raised against both heavy and light chains (H+L)	Goat IgG	IHC 2° 1:300	Vector Laboratories, Inc., Burlingame, CA (BA-1000)

FC, Flow cytometry; **IHC**, Immunohistochemistry; **1°**, primary antibody; **2°**, secondary antibody.

Table 2-2. PCR Primers

Target gene name	Primer sequence	Amplicon size (bp)	Annealing temperature
Human *CDH5*	Fwd: TGACAATGTCCAAACCCACTCA Rev: TGACAACAGCGAGGTGTAAAGAC	72	60°C
Human *KDR*	Fwd: ATCACACAATTAAAGCGGGG Rev: CTGGGGTGGGACATACA	149	60°C
Human *EPHA2*	Fwd: AGACGCTGAAAGCCGGCTAC Rev: CAGGGCCCCATTCTCCATG	164	60°C
Human *LAMC2*	Fwd: AACCCACAACCCACAACCTT Rev: GCCGAAAGTTATCTGGGCCT	147	57°C
Human *MMP1*	Fwd: CTGGCCACAACTGCCAAATG Rev: CTGTCCCTGAACAGCCCAGTACTTA	103	57°C
Human *MMP2*	Fwd: TTTCCATTCCGCTTCCAGGGCAC Rev: TCGCACACCACATCTTTCCGTCACT	253	57°C
Human *MMP9*	Fwd: CTATTTCTGCCAGGACCGCT Rev: GTTGGTCCCAGTGGGGATTT	162	57°C
Human *MMP14*	Fwd: GGAGAATTTTGTGCTGCCCG Rev: TTGGTTATTCCTCACCCGCC	247	57°C
Human *GAPDH*	Fwd: CCATGTTGCAACCGGGAAG Rev: CATCACCCGGAGGAGAAATCG	139	60°C
Human *ACTB*	Fwd: ATGTACGTTGCTATC Rev: CTCCTTAATGTCACG	249	60°C
Human *CYCA*	Fwd: GGCAAATGCTGGACCCAACACAAA Rev: CTAGGCATGGGAGGGAACAAGGAA	335	60°C
Mouse *Cox1*	Fwd: TTGCACAACACTTCACCCACCAG Rev: AAACACCTCCTGGCCCACAGCCAT	208	59°C
Mouse *Cox2*	Fwd: GGAGAGAAGGAAATGGCTGCA Rev: ATCTAGTCTGGAGTGGGAGG	363	59°C
Mouse *Hrpt1*	Fwd: CCCAGCGTCGTGATTAGCG Rev: GCACACAGAGGGCCACAATG	187	59°C
Mouse *CycA*	Fwd: AGCGTCCCTACCCCATCTG Rev: AGGAAAATATGGAACCCAAAGAACT	77	59°C

Fwd, Forward primer; **Rev**, Reverse primer; **bp**, base pairs. Annealing temperature indicates temperature used in PCR and qPCR unless otherwise indicated.

Table 2-3. Cell culture reagents

Name	Description	Source (catalogue #)
Baxter Water	Sterile Baxter Water for irrigation	Baxter Healthcare, Regency Park, SA, Australia (AHF7114)
Collagenase (TYPE I)	Enzyme which liberates ECs from the umbilical vein (Appendix A1-1)	Worthington Biochemical Corporation, Lakewood, NJ, USA (4197)
DMEM	Dulbecco's Modified Eagle's cell culture medium	Life Technologies, Carlsbad, CA, USA (11995)
Endothelial Cell Growth Supplement	Cell culture medium supplement (includes Fibroblast Growth Factor and Endothelial Cell Growth Factors α and β)	BD Biosciences, San Jose, CA, USA (BD356006)
FBS	Foetal Bovine Serum	Bovogen, Keilor East, VIC, Australia (SFBS)
Gelatin	Cell culture flask coating to improve cell attachment (Appendix A1-5)	Sigma Aldrich, St Louis, MO, USA (G9391)
Heparin	Heparin Sodium Salt, bovine	Sigma Aldrich, St Louis, MO, USA (H-0777)
1M HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Cell culture medium buffering agent	Life Technologies, Carlsbad, CA, USA (15630)
M199	M199 cell culture medium	Sigma Aldrich, St Louis, MO, USA (M4530)
Non-essential amino acids (100x)	Cell culture medium supplement	Sigma Aldrich, St Louis, MO, USA (M7145)
10x PBS	Sterile Phosphate buffered saline	Life Technologies, Carlsbad, CA, USA (AM9625)
Penicillin Streptomycin solution	Antibiotic solution for cell culture medium	Life Technologies, Carlsbad, CA, USA (15140)
RPMI	Roswell Park Memorial Institute cell culture medium	Life Technologies, Carlsbad, CA, USA (11875)
Sodium bicarbonate	Cell culture medium buffering agent	Sigma Aldrich, St Louis, MO, USA (S8761)
100mM Sodium Pyruvate	Cell culture medium supplement	Life Technologies, Carlsbad, CA, USA (11360-070)
Trypsin (2.5%)	Digestive enzyme for harvesting cells from culture flasks	Gibco, Life Technologies, Carlsbad, CA, USA (15090-046)

Table 2-4. Molecular reagents

Name	Source (catalogue #)
CD36 Trilencer-27 Human SiRNA	OriGene Technologies, Rockville, MD, USA (SR300671)
Deoxynucleotide (dNTP) Solution Mix	New England Biolabs, Beverly, MA, USA (N0447L)
Gel Red™ (10 000x)	Biotium Inc, Hayward, CA, USA (41003)
QuickLoad 50bp DNA ladder	New England Biolabs, Beverly, MA, USA (N0473S)
QuickLoad 100bp DNA ladder	New England Biolabs, Beverly, MA, USA (N0467S)
Lipofectamine RNAiMAX	Life Technologies, Carlsbad, CA, USA (13778-150)
Opti-MEM® Reduced Serum Medium	Thermo Fisher Scientific, Waltham, MA, USA (31985070)
QIAprep Miniprep Spin Columns	Qiagen, Hilden, Germany (27115)
Phusion® High-Fidelity DNA Polymerase	New England Biolabs, Beverly, MA, USA (M0530)
QIA Shredder Column	Qiagen, Hilden, Germany (79654)
QuantiTect SYBR® Green Master Mix	Qiagen, Hilden, Germany (204145)
Rneasy Plus Mini kit	Qiagen, Hilden, Germany (74134)
SuperScript® III Reverse Transcriptase	Life Technologies, Carlsbad, CA, USA (18080-093)
TRIzol® Reagent	Life Technologies, Carlsbad, CA (15596026)

Table 2-5. Miscellaneous reagents and consumables

Name	Description	Source (catalogue #)
7-AAD staining Solution	7-aminoactinomycin D. Used for flow cytometry staining at 1:5 dilution to detect and exclude dead cells from analysis	BD Biosciences, San Jose, CA, USA (559925)
Acetylsalicylic Acid	Aspirin, platelet aggregation inhibitor. Reconstituted in EtOH at 25mg/mL and diluted with corn oil at 2.5 mg/mL.	Sigma Aldrich, St Louis, MO, USA (A5376)
Apyrase	Apyrase (ATP and ADP degrading enzyme), reconstituted in MQ H_20 at 100 U/ml.	BD Biosciences, San Jose, CA, USA (A6132)
Human α-thrombin	100 IU purified human α-Thrombin	Hyphen Biomed, Neuville-sur-Oise, France (EZ006A)
Corn oil	Vehicle for *in vivo* studies	Sigma Aldrich, St Louis, MO, USA (C8267)
D-Luciferin	Chemiluminescent substrate of firefly luciferase. Reconstituted in PBS (Appendix A1-10) at 30 mg/mL (10x)	Cayman chemical, Ann Arbor, MI, USA (14681)
DMSO	Dimethyl sulfoxide, cryoprotective agent.	Sigma Aldrich, St Louis, MO, USA (D2650)
DPX Mountant for histology	DPX Mountant for histology is a mixture of distyrene, a plasticizer, dissolved in toluene-xylene and used as a synthetic resin mounting media	Sigma Aldrich, St Louis, MO, USA (06522)
FcR Blocking Reagent, human	block non-specific binding of antibodies to human Fc receptor-expressing cells	Miltenyi Biotech, Bergisch, Gladbach, Germany (130-059-901)
Gavage needles	20G-38mm soft (bulbed) tip feeding tubes	Instech laboratories, Plymouth Meeting, PA, USA (FTP-20-38)
Geltrex Matrix	Soluble reduced growth factor basement membrane extract purified from murine Engelbreth-Holm-Swarm tumour.	Thermo Fisher Scientific, Waltham, MA, USA (A1413202)
ImmPACT™ DAB (Brown)	Peroxidase substrate solution	Vector Laboratories, Inc., Burlingame, CA (SK-4105)

In Situ Cell Death Detection Kit, Fluorescein	Allows for the identification of apoptosis at a molecular level (DNA-strand breaks) and identification of cells at the very early stages of apoptosis (TUNEL)	Roche Diagnostics, Indianapolis, IN, USA (11684795910)
Liquid DAB+ Substrate Chromogen System	Immunohistochemical staining; 3,3'-diaminobenzidine in chromogen solution with Imidazole-HCl buffer. DAB diluted 1:50 in buffer.	Dako, Glostrup, Denmark (K3468)
Lysing Matrix D, 2 mL Tube	Homogeniser tubes containing 1.4 mm ceramic spheres for rapid sample lysis.	MP Biomedicals, Santa Ana, CA, USA (116913050)
Matrigel® Basement Membrane Matrix	Solubilized basement membrane extract from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma	Corning Life Sciences, New York, USA (354234)
Microcentrifuge tubes, extended capacity	1.7mL, graduated, siliconized polypropylene. Used to ensure produce recovery of thrombospondin.	Sigma Aldrich, St Louis, MO, USA (605225)
Normal Goat Serum	Used as a blocking agent in IHC	Sigma Aldrich, St Louis, MO, USA (G9023)
PAS staining kit	Periodic acid Schiff reagent. As per manufacturers instruction, used to detect polysaccharides and mucosubstances (including basement membrane).	Merck Millipore, Billerica, MA, USA (101646)
Polybead (2µm) Polystyrene Microspheres	Monodisperse polystyrene microspheres, 2.00µm mean diameter, provided at 5.68×10^9 particles/mL	Polysciences Inc., Warrington, PA, USA (19814-15)
Dako REAL Detection System, Peroxidase/DAB+ kit	Including Streptavidin Peroxidase (HRP) solution, DAB+Chromogen (X 50) solution, and HRP substrate buffer	Dako, Glostrup, Denmark (K5007)
Thrombospondin, Human platelets	Ligand to CD36 receptor.	Merck Millipore, Billerica, MA, USA (605225).
VECTASHIELD Antifade Mounting Medium with DAPI	Mounting Medium which prevents rapid photobleaching of fluorescent proteins and fluorescent dyes	Vector Laboratories, Inc., Burlingame, CA (H-1200)
VECTASTAIN elite ABC reagent	Immunoperoxidase staining system	Vector Laboratories, Inc., Burlingame, CA (PK-7100)

ADP, adenosine diphosphate; **ATP**, adenosine triphosphate; **MQ H_20**, Milli Q water; **EtOH**, ethanol; **IHC**, Immunohistochemistry; **IU**, International units.

2.3 Mammalian cell culture

2.3.1 Human umbilical vein endothelial cell (HUVEC) isolation

HUVECs were isolated from human umbilical cords by collagenase digestion as previously described (Litwin et al. 1997; Moldenhauer et al. 2015; Wall et al. 1978). Human umbilical veins were collected and inspected for any tears or holes. Cords of >20cm in length were used for EC extraction. The umbilical vein was distended and an adaptor with a stopcock was attached to the vein and tied with twine. The vein was filled with PBS (Appendix A1-10) and checked for any leakage or holes. Intact veins then had an adapter with a stopcock attached to the other end. The vein was then flushed out before filling with collagenase (Appendix A1-1) The vein was placed in a beaker filled with 0.9% saline (Appendix A1-2) for 7 min at 37°C (this timing ensures that only ECs were isolated). After incubation, syringes were attached to both stopcocks and one end was flushed with RPMI, allowing for the collection of the collagenase/cells into the other syringe. Collagenase/cell solution was separated evenly into 3x50mL tubes. The vein was refilled with RPMI and massaged to remove any remaining cells, and media was collected. The vein was flushed twice more. Collected collagenase/cells were centrifuged at 400xg for 5 min at room temperature (RT) and washed twice in HUVEC wash (Appendix A1-4) before being added to gelatin (Appendix A1-5) coated culture flasks at 37°C in 5% CO_2. Media was replaced every 2 days. In preparation for harvesting, EC cultures were washed with 0.01M EDTA/PBS (Appendix A1-7, A1-8 and A1-9) incubating with 0.05% trypsin solution (Appendix A1-11). Cells were detached by gently tapping the flask, then collected in complete HUVEC media (Appendix A1-3) and centrifuged at 400xg for 5min at RT. The EC pellet was resuspended in 8mL fresh HUVEC medium and seeded into gelatin coated flasks. Once ECs were split from their

primary flask, heparin (Appendix A1-6) and endothelial growth factors (Table 2-3) were added to the media (3µl per 1ml HUVEC media). HUVECs were used in experiments at no later than the second passage.

2.3.2 **Human cancer cell lines**

Human cancer cell lines and respective culture conditions are described in Table 2-6. Cells were cultured in T75 flasks and passaged when they reached 80-90% confluence. To harvest the cells, media was removed and 0.01M EDTA/PBS was added to flasks which were then incubated for 5 min at 37°C. Cells were detached by gentle tapping and collected with complete DMEM or RPMI medium before being centrifuged at 400xg for 5min at RT. Cell pellets were resuspended in fresh complete medium, and split into new flasks at dilutions 1:5-1:20. Split cells were incubated at 37°C in 5% CO_2. Cells were never passaged for more than 2 months.

2.3.2.1 ***Freezing and thawing cell lines***

Cancer cell lines from 1 x T75 flask were harvested as described (Section 2.3.2) and centrifuged at 400xg for 5 min at RT. Trypan blue (Appendix A1-12) was added to an aliquot of the suspension and viable cells counted in a haemocytometer. Cold freeze mix (800 µL of 10% DMSO in 90% FBS) was added for every $1x10^7$ cells. Resuspended cells were aliquoted into cryovials ($1x10^7$ per vial) and placed in a Mr Frosty™ Freezing container (Thermo Scientific, Waltham, MA, USA) for 24 hours at -80°C, before being transferred into liquid nitrogen (LN_2) for long term storage. To recover cells, vials were retrieved from LN_2 storage and thawed rapidly in a water bath a 37°C. Vial contents were transferred to a 50mL tube and 2mL cold RPMI (for melanoma cell lines) or cold DMEM (for breast cancer cell lines) was added drop wise. An additional 8mL of cold complete

medium was added to the tubes while mixing. Resuspended cells were kept at RT for 10 min before centrifuging at 400xg for 5 min at RT. Cell pellets were resuspended in fresh warm (37°C) complete medium and transferred to a new flask. Flasks were incubated at 37°C in 5% CO_2.

Table 2-6. Human cancer cell lines

Name	Description	Growth conditions	Source
C32	Skin amelanotic melanoma, adherent	RPMI + 10% FBS	ATCC, Manassas, Virginia, USA (CRL-1585)
CHL-1	Skin epithelial melanoma, adherent	RPMI + 10% FBS	ATCC, Manassas, Virginia, USA (CRL-9446)
HS-578T	Breast epithelial carcinoma, adherent	DMEM + 10% FBS	ATCC, Manassas, Virginia, USA (HTB-126)
MDA-MB-231	Breast epithelial adenocarcinoma, adherent	DMEM + 10% FBS	ATCC, Manassas, Virginia, USA (HTB-26)
MDA-MB-231-LM2	A highly metastatic firefly luciferase-tagged variant of MDA-MB-231.	DMEM + 10% FBS	Provided by Dr Joan Massagué

2.3.3 **Human platelet isolation**

2.3.3.1 ***Collection of peripheral blood***

Venous blood was collected from individuals between the ages of 18 and 65 years who were non-smokers, aspirin/NSAIDs free for at 1 month, and did not have any blood disorders. Whole blood was collected primarily from the median cubital vein (or the cephalic vein) using a 21G needle butterfly set. To avoid thrombin generation in the sample, the first 1-2ml of blood was collected into a syringe and discarded. The remainder of the blood was collected into a fresh syringe which was immediately transferred to a tube containing acid-citrate dextrose (ACD) anticoagulant (Appendix A1-13). Six parts blood was added to one part ACD and gently mixed [16.6% vol/vol].

2.3.3.2 ***Platelet washing procedure***

Platelets were purified as described elsewhere (Brockmann et al. 2011; Cazenave et al. 2004; Chang et al. 2015; Mustard, Kinlough-Rathbone & Packham 1989; Mustard et al. 1972). Briefly, collected blood was left to rest at RT for 15 min before centrifugation at 180xg for 10 min with no brake. Platelet-rich-plasma was carefully collected into a separate tube and centrifuged at 1,100xg for 15 min with no brake. The supernatant was discarded and excess plasma was wiped away with cotton swabs before re-suspending the platelet pellet gently with Tyrode's platelet wash buffer (Appendix A1-16). The platelet suspension was then incubated at 37°C for 5 min before centrifugation at 1,100xg for 15 min. Supernatant was discarded and the platelet pellet was resuspended in Tyrode's platelet resuspension buffer (Appendix A1-14, A1-15 and A1-17). Platelets were then counted using a Sysmex XE-5000 differential analyzer (Sysmex Corporation, Kobe, Japan) and counts were adjusted to appropriate concentrations.

2.3.4 **Collection of platelet-released contents**

Supernatant (containing platelet-released contents) from activated platelets was prepared as described previously (Labelle, Begum & Hynes 2011). Samples of washed platelets were adjusted to ~100x10^6/mL and treated with 0.5 International Units (IU) human α-thrombin (Table 2-5) for 10 min to induce platelet activation. To confirm platelet activation, CD62P expression was measured by flow cytometry (section 2.4.1). Samples were centrifuged (2,800xg for 10min) and supernatant was collected and stored at -20°C for later use in co-culture tube formation studies. Control samples contained the same concentration of thrombin as test samples. Supernatant was used in the VM tube formation assay (cancer cell: supernatant) at the ratios of 1:0 (control), 1:2, 1:5, 1:10, 1:20, and 1:40, the volume corresponding to the number of platelets the supernatant was collected from.

2.3.5 ***In vitro*** **tube formation assay**

The Matrigel tube formation assay was used to assess angiogenesis or VM by ECs or cancer cells, respectively, in the presence or absence of platelets. Basement membrane extract (BME) Geltrex (Table 2-5) was left to thaw on ice at 4°C overnight. The following day, Angiogenesis μ-slides (Ibidi, Munich, Germany) culture wells were coated with 10ul of BME and incubated at 37°C for 30 min to allow for solidification. HUVECs were harvested as previously described in section 2.3.1. Cancer cells were harvested as previously described in section 2.3.2. Platelets were purified as previously described in section 2.3.3.

HUVECs were plated at an optimal seeding concentration of 1x10^4 cells per well (Moldenhauer et al. 2015), or sub-optimal seeding concentration of 5x10^3 per well, determined for these experiments.

Cancer cell numbers for seeding concentrations were determined previously by Ms Emma Thompson in the Vascular Biology and Cell Signalling Laboratory (Centre for Cancer Biology), and verified in this work (data not shown). The following number of cells per well were used; C32 melanoma, $2x10^4$ cells; CHL-1 melanoma, $1.5x10^4$ cells; MDA-MB-231 breast cancer, $1.5x10^4$ cells; and HS-578T breast cancer, $1.5x10^4$ cells. Sub-optimal seeding concentrations (the lowest concentration of cells wherein tube structures were still formed) of cancer cells were optimised for these experiments and were: C32 melanoma, $1.5x10^5$ cells; and HS578T breast cancer, $7.5x10^4$ cells. For co-culture studies, platelet concentration was adjusted with Tyrode's wash buffer to result in a 2-fold, 5-fold, 10-fold, 20-fold, or 40-fold concentration of platelets (compared to cell number) in 10µl volume, based on studies by Pipili-Synetos and colleagues (1998). Herein, concentrations are referred to as ratios of 1:2, 1:5, 1:10, 1:20, and 1:40 (cells: platelets). HUVEC or cancer cell concentrations were adjusted to contain the seeding concentrations per 40uL culture medium. Platelets were mixed with HUVECs or cancer cells before seeding in duplicate into Geltrex-containing wells and incubated at 37°C in 5% CO_2. After 5 hours (cancer cells) or 8 hours (HUVECs) of incubation, images of tube formation were captured using an Olympus CKX-41 Inverted Microscope (Tokyo, Japan) with 4x objective, fitted with a DP70 digital camera and operated through the Olympus Image Analysis Cell B software program (Olympus, Tokyo, Japan). Tube formation was quantified by counting the number of tube-like structures per well using Image J software (National Institute of Health, USA). These structures were defined as elongated arrangements of cells that are tightly aligned and extend between collections of cells. In other experiments, platelets were substituted with platelet-sized beads (Table 2-5), or with supernatant collected from α-thrombin activated platelets (section 2.3.4). For time-lapse

video microscopy, images were captured using a 10x objective on a CellVoyager™ CV1000 Confocal Scanner System and software (Yokogawa, Tokyo, Japan).

2.4 Flow cytometric analysis

2.4.1 Platelet validation

To assess platelet populations for purity, 95µL samples of purified washed platelets (~100x10^9/L) were stained with 5µL FITC-conjugated anti-human CD42b antibody or mouse IgG isotype control (Table 2-1) for 15 min in the dark. To assess for activation the platelet population was stained with 5µL PE-conjugated anti-human CD62P antibody or 5µL mouse IgG isotype control (Table 2-1) for 15 min in the dark. Samples were then diluted with PBS (300uL) and measured using a BD Accuri C6 flow cytometer (Ann Arbor, MI, USA) with subsequent analyses performed on FCS Express 6 cytometry software (De Novo Software, Glendale, CA).

2.4.2 Cancer cells

Cancer cells were assessed for cell surface expression of various markers by flow cytometry. Cancer cells were harvested and Fc receptors were blocked with human IgG (Table 2-5) (1:50 in HUVEC wash medium) for 10 min. A sample of 1x10^6 cells was incubated with primary antibodies (Table 2-1) or relevant isotype controls in a final volume of 100uL. Cells were washed with 1mL HUVEC wash medium and centrifuged at 400xg for 5 min before incubating with goat anti-mouse DyLight650 (Abcam, Cambridge, UK) (Table 2-1) for 30 min on ice. In the last 5 min of incubation, 5µl of 7-AAD (Table 2-5) was added to each tube. Cells were washed again and resuspended with 300µL FACS fixative (Appendix A1-18). Data was collected using a BD Accuri C6 flow

cytometer (Ann Arbor, MI, USA) and analysis was performed on FCS Express 6 cytometry software (De Novo Software, Glendale, CA).

2.5 Molecular techniques

2.5.1 RNA extraction

Frozen halves of tumours were partially thawed and placed in Lysing Matrix D homogeniser tubes (Table 2-5) with 1mL Trizol reagent (Table 2-4). Samples were homogenised on MP FastPrep®-24 instrument (MP Biomedicals, Santa Ana, CA, USA). Total RNA was extracted using the RNeasy Mini Plus Kit (Table 2-4), as per the manufacturer's instructions. RNA was eluted in RNase-Free Water and concentration determined using the NanoDrop-100 Spectrophotometer (Thermo Fisher Scientific, MA, USA). Total RNA was stored at -80˚C until cDNA synthesis.

2.5.2 cDNA synthesis

A sample of 0.5-1µg of total RNA was converted into first strand cDNA using Superscript III reverse transcriptase (Table 2-4) following manufacturer's instructions. Reactions were run on a thermocycler (Biorad); 30 min at 50°C, 30 min at 55°C followed by 15 min at 72°C. The 30µl cDNA reaction was then diluted in 170µl TE Buffer (Appendix A1-19) and stored at -20˚C until used for quantitative PCR (qPCR).

2.5.3 Polymerase chain reaction (PCR) and agarose gel electrophoresis

PCR was performed using Phusion® High-Fidelity DNA Polymerase with HF Buffer (Table 2-4). Each PCR reaction contained 0.1µL (0.2U) Phusion DNA Polymerase, 4µL 5x HF Buffer (NEB), 0.6uL DMSO, 0.5uL 10mM dNTPs, 1µl 100ng 5' and

3'oligonucleotide and 2µl (1ng) cDNA template. PCR reaction parameters were run as stated: 2 min at 98˚C, followed by 34 cycles at 98˚C for 20 seconds, annealing temperature (Table 2-2) for 20 seconds and 72˚C for 30 seconds and finally 72˚C for 5 min on a thermocycler (Bio-Rad).

DNA products were run on agarose gels (Appendix A1-20) for qualitative assessment of the PCR reaction (Table 2-2). Agarose gels (1.5%) were prepared by dissolving agarose in 1x Tris-acetate-EDTA (TAE) buffer (Appendix A1-21) by brief warming in a microwave. Before pouring gel into moulds, 2µl 3xGel Red in 0.1M NaCl (Biotium, California, USA) was added to agarose. Agarose gels were poured and allowed to set at RT for 10 min. DNA samples were electrophoresed at 120V for 45 min. The QuickLoad 50bp or 100bp DNA ladder (NEB) was used to estimate DNA fragment size. DNA fragments were visualised under ultraviolet (UV) light using Uvitec gel documentation system (Uvitec, Cambridge, UK).

2.5.4 **Quantitative Real Time PCR**

All reactions were completed in a 20 µL reaction volume in triplicate. Sample preparation was automated using the QIAgility automated PCR setup instrument (Qiagen, Hilden, Germany). Samples were loaded onto 100-well rotors on Rotor-Gene thermocycler (Corbett Research, Mortlake, NSW, Australia). Reaction parameters began with a 2 min hold at 50°C, followed by a 15 min hold at 95°C. Then, a total of 45 cycles were performed as follows; 10 sec at 95°C, 20 sec at annealing temperature (Table 2-2), 30 sec at 72°C. Data analysis was performed using Rotor-Gene Analysis Software version 6 (Corbett Research). Relative gene expression levels were calculated using the comparative quantitation method available in the Rotor-Gene Software. The software determines the 'take-off' point of a reaction and the slope of the line from the take-off

point (until exponential amplification stops) is used to calculate the amplification efficiency (Qiagen). Genes expression was normalised against mouse *Hrpt* and *CycA* for livers, and human *GAPDH*, *ACTB*, and *CYCA* for tumours (Table 2-2). The suitability of the mouse and human housekeeper genes was validated using the GeNorm (M value < 1.5) software package (Vandesompele et al. 2002).

2.5.5 **Protein knockdown with small interfering RNA gene silencing**

Cell transfections with small interfering RNA (siRNA) were performed in T25 flasks and 12-well plates. Cancer cells were harvested from T75 flasks when they reached 80% confluency. Cells were then centrifuged at 400xg and resuspended in complete 800µL culture medium to achieve 10% of the cells per 100µL. For transfection into T75 flask, 133.33 µL cells were made up to 6mL with culture medium, or for transfection into a 12-well plate, 121.6µL cells were made up to 6mL with culture medium. Stock concentrations (20µM) of siRNA were diluted with Opti-MEM® Medium to 606nM. For the formation of siRNA-lipid complexes, 5uL of RNAiMAX was incubated with 10µL 606nM siRNA in 990µL Opti-MEM® Medium and incubated at RT for 15 min. After incubation, 1mL of the siRNA-lipid complex was added to the cells in 5mL culture medium. A total of 6mL was used for transfections in T25 flasks, and 1.2mL/well was used for 12-well plate transfections. Plates and flasks were incubated for 48 hours at 37°C in 5% CO_2. Knockdown at the protein level was validated by flow cytometric analysis (section 2.4.2).

2.6 *In vivo* methods

2.6.1 *In vivo* mammary fat pad injections

Female NOD/SCID mice (6-8 weeks of age) were housed in the SA Pathology Animal Care Facility for 1 week before orthotopic injections. Mice were anesthetised before being injected with MDA-MB-231-LM2 breast cancer cell line which stably express firefly luciferase (Minn et al. 2005). Using a 0.1ml 26 gauge needle, $1x10^6$ cells in 50µL of 50% Matrigel were injected subcutaneously into the mammary fat-pad (MFP) (Table 2-5). Tumours were allowed to develop for 7 days before mice were randomly allocated into two groups (n=11 per group) using tail markings as an identification method. Tumour size (determined by caliper) and mouse weight was measured every 2-3 days. Tumour volume was calculated using the following formula; Volume of ellipse (mm^3) = [length x $width^2$]/2.

2.6.2 Gavage procedure

After allowing tumours to develop for 7 days, mice were divided into two groups (n=11 per group). Each group was then treated daily with either vehicle or aspirin (25mg/kg in corn oil; Table 2-5) by gavage. The mouse dose equivalent to 100-150mg/60kg human low dose aspirin was calculated using the following formula;

$Human\ equivalency\ dose\ (HED) = animal\ dose\ \left(\frac{mg}{kg}\right)$ x $\frac{Animal\ Km}{Human\ Km}$, where mouse *Km* factor is 3, and human *Km* factor is 37 (Reagan-Shaw, Nihal & Ahmad 2008). The *Km* value is a constant based on average BSA calculations which depends on the species, increasing as weight increases (U.S. Food and Drug Administration 2005).

For a 25 mg/kg dose, aspirin was prepared at 2.5 mg/mL (Doutremepuich et al. 2012) (Appendix A1-22) and solution was loaded into a 1mL syringe fitted with a 20G-38mm

gavage needle (Table 2-5). Each mouse received 10 µl/g body weight to deliver a dose of 25 mg/kg. Control group received the same volume of ethanol in corn oil. Drug was administered daily and volume was adjusted depending on the weight of mouse. Each mouse was monitored until fully recovered after each gavage.

2.6.3 **Bioluminescence imaging**

Bioluminescence imaging (BLI) was carried out weekly by administering luciferin peritoneally and measuring total flux (photons/second; p/s). Each mouse received a dose of 3mg of luciferin in 200ul PBS (Appendix A1-23) into the peritoneal cavity using a 25 gauge needle. In this way, cancer cells expressing luciferase enzyme can cleave the protein and produce luminescence. Whole-animal BLI was performed using the Xenogen IVIS 100 (Caliper Life Sciences, Hopkinton, MA, USA) available at Adelaide Microscopy (Adelaide, Australia). Imaging was performed 10 min after injections. Living Image software (PerkinElmer, Waltham, MA, USA) was used to quantify primary mammary fat pad tumour development. Images were obtained weekly until the end of the study. On the final day of the study, mice were humanely killed. Primary tumour, lung, liver, hind limbs and spine were harvested to be imaged *ex vivo* to detect metastases. After imaging, primary tumours were fixed in 10% buffered formalin (Appendix A1-24) for 24 hours before routine processing and embedding in paraffin wax by SA pathology (Adelaide, SA, Australia). Other organs were frozen using dry ice and stored at -80°C.

2.6.4 **Haematoxylin and eosin (H&E) staining**

Tissue sections (cut at 4μm) were deparaffinised and rehydrated before being stained with haematoxylin (HX) and eosin (both from Dako, Glostrup, Denmark). Briefly, sections were incubated with Ehlrich's HX (1 min) and stained with eosin (1 min) before dehydration, clearing and mounting with DPX medium (Sigma Aldrich, MO, USA) (Table 2-5).

2.6.5 **Immunohistochemistry (IHC) staining**

IHC was performed on human blood smears and human spleen sections (positive control tissue sourced from University of South Australia, Adelaide, SA, Australia) to test the specificity of CD41 (platelet GPIIb) antibody, on harvested xenograft tumour sections for CD41 expression, and on tumours sections for CD31/PAS expression. Tissue sections were deparaffinised and rehydrated as described above prior to IHC staining. Images were captured using an Olympus BX40 microscope fitted with a DP70 digital camera and operated through the Olympus Image Analysis Cell B software program (Olympus, Tokyo, Japan).

2.6.5.1 ***CD41 antibody; whole blood smears***

Whole blood was collected from donors as previously described (Section 2.3.3.1). Blood smears were prepared on glass slides and air dried before being fixed in 100% methanol for 5 min. Antigen retrieval was not performed on fresh blood smears. Endogenous peroxidase was quenched with 3% H_20_2 (Appendix A1-26) for 10 min. Sections were washed for 2 min in tris buffered saline (TBS; Appendix A1-27, A1-28 and A1-29) before being blocked for 10 min with 3% non-immune goat serum (IMVS Veterinary Services, Northfield, Australia). Excess solution was tapped off and smears were incubated

overnight at 4°C with anti-CD41 antibody (Table 2-1) diluted to 1/800 with TBS. Negative control sections were incubated with TBS alone. Sections were washed the following day under a stream of TBS and rinsed twice for 2 min each in TBS. Sections were incubated for 30 min with goat anti-rabbit biotinylated antibody (Table 2-1) then washed three times for 2 min each with TBS. Staining was achieved using the labelled streptavidin-biotin method (LSAB). Briefly, excess buffer was tipped off and sections were incubated with 3-4 drops of streptavidin-HRP reagent (Table 2-5) for 10 min in a humid chamber. Sections were then rinsed three times for 2 min each with TBS. Sections were then incubated with DAB + substrate-chromogen (Table 2-5) for 10 min. Sections were washed for 2 min in water and counterstained with Ehlrich's HX (1 min), rinsed in water, differentiated in acid alcohol, washed, blued in ammonia water (1 min) (Appendix A1-25). Sections were then dehydrated, cleared and mounted as described in section 2.6.4.

2.6.5.2 *CD41 antibody; paraffin sections*

Human spleen and mouse xenograft tumour sections were deparaffinised and rehydrated as described in section 2.6.4. Heat induced epitope retrieval was performed using citrate solution (Appendix A1-30, A1-31 and A1-32) adjusted to pH 6.0. Sections were immersed in citrate retrieval solution (250mL) and placed in a Sharp model (R-9270) microwave oven (Tokyo, Japan). An additional container filled with equal volume of distilled water was placed in the microwave oven to act as a heat balance. The solutions were then heated on HIGH for 4 min (boiling point) followed by 10 min on MEDIUM-LOW (simmer). The solution was then cooled to RT by submerging in a water bath. Once cooled, sections were washed with TBS for 5 min. Sections were then quenched with 3% H_20_2 for 5 min followed by rinsing in distilled water. Excess water around sections was

then carefully wiped with tissue and sections were encircled with PAP pen (Agilent, Victoria, Australia). Sections were washed 5 times for 2 min each in TBS before being blocked for 10 min with 3% non-immune goat serum. Blocking solution was then tapped off and sections were incubated for 1 hour with anti-CD41 antibody in a humid chamber. Sections were then washed for 2 min in TBS (5 times) before incubation with goat anti-rabbit biotinylated antibody (Table 2-1) for 30 min. IHC staining was then developed using the LSAB method (described in section 2.6.5.1) and sections were dehydrated, cleared and mounted as described in section 2.6.4.

2.6.5.3 *CD31 antibody and PAS staining*

Tissue sections of xenograft mouse tumours were deparaffinised and rehydrated as described in section 2.6.4. Heat induced epitope retrieval was performed using citrate buffer (Appendix A1-34). Sections were immersed in citrate retrieval solution (250mL) and placed in a Samsung ME6124W-1 microwave oven (Seoul, South Korea) with an additional container filled with equal volume of distilled water to act as a heat balance. Sections were microwaved at 900W for 4 min, followed by 20 min 350W. Sections were then allowed to cool to RT. Quenching was performed using 1% H_20_2 (Appendix A1-33) for 10 min followed by washes in distilled water (3x 5 min) and one 5 min wash in PBS. Sections were blocked with 10% normal blocking serum (Table 2-5) diluted in PBS for 1 hour. Excess solution was blotted and sections were incubated overnight with anti-CD31 primary antibody (Table 2-1) diluted with 3% normal serum in PBS. The following day sections were washed three times for 5 min each in PBS before incubating with goat anti-rabbit biotinylated antibody for 35 min (Table 2-1). Sections were again washed three times for 5 min each then then incubated with VECTASTAIN elite ABC reagent (Table 2-5) for 30 min. Reagent was prepared by mixing 2 drops of Reagent A in 5mL buffer,

followed by the addition of 2 drops of Reagent B, and allowing the mixture to stand for 30 min before use. Sections were then washed three times for 5 min each in PBS before incubating for 2-10 min in peroxidase substrate (Table 2-5) solution. Solution was prepared by adding 2 drops of buffer stock solution, 4 drops of DAB, and 2 drops of hydrogen peroxide solution into 5mL water. Sections were then rinsed well in tap water. PAS staining was performed on sections following manufacturer instructions (Merck Millipore, MA, USA) (Table 2-5). Sections were then dehydrated, cleared and mounted as described in section 2.6.4.

2.6.6 Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay

Sections of xenograft mouse tumours were deparaffinised and rehydrated as described in section 2.6.4. Slides were then placed in a plastic jar containing citrate buffer (Appendix A1-35). Antigen retrieval was performed using a Samsung ME6124W-1 microwave oven (Seoul, South Korea) by microwaving at 750W for 1 min. Sections were cooled rapidly by immediately adding 80mL of distilled water then immersed into Tris-HCl (Appendix A1-36 and A1-37) for 30 min at RT. Sections were washed twice with PBS. Excess fluid was tapped off and 50µl of TUNEL reaction mixture (Table 2-5) was added to the section and incubated for 60 min at 37°C in a humidified atmosphere in the dark. Sections were then rinsed three times in PBS for 5 min each. Vectashield with DAPI (4',6-diamidino-2-phenylindole) was used to mount slides which were then left to dry overnight on the bench protected from light. Sections were imaged using the 3DHistech Panoramic Confocal system and analysed with Panoramic Viewer software (3DHistech Ltd., Budapest, Hungary). The percent area of TUNEL positivity was measured using ImageJ software (National Institute of Health, USA). This was calculated by firstly quantifying the total

area of the tumour by drawing around the border of DAPI fluorescence channel, then upper and lower thresholds for 8-bit image were adjusted to determine total fluorescence in the green channel. The following formula was used to calculate percent positive TUNEL staining, (TUNEL+ area / total tumour area) x 100.

2.7 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM), or with median line, from at least 3 experiments. Statistical analysis was performed using GraphPad Prism software (San Diego, CA, USA). One-way ANOVA, Mann Whitney U test or Student's t-test was performed to determine statistical significance, with *P* values <0.05 considered significant.

Chapter 3. Platelet role towards VM-formation in breast cancer and melanoma *in vitro*

3.1 Introduction

Cancer is one of the leading causes of death and morbidity worldwide (Ferlay J 2012), with both breast cancer and melanoma being amongst the most significant in terms of mortality in Australia (Cancer Australia). It is well established that the growth of malignant tumours beyond 1-2mm^3 is dependent on tumour angiogenesis, a process wherein pre-existing normal vessels are directed to the growing tumour (Folkman 1971, 1990). Angiogenesis research has identified the importance of various growth factors in this process (discussed in detail in Chapter 1), including VEGF, PlGF, PDGF, and bFGF (Falcon et al. 2016) with VEGF signalling in particular playing a pivotal role (detailed in chapter 1) (Hillen & Griffioen 2007). It is known that VEGF is released from various sources including platelets (Blair & Flaumenhaft 2009).

3.1.1 Platelets in angiogenesis

The earliest evidence suggesting a promotive role of platelets in angiogenesis was demonstrated using the *in vitro* angiogenesis assay. In this assay, HUVECs are cultured on a matrix of basement membrane extract proteins (Matrigel) and angiogenic potential is assessed by quantifying the number of vascular-like tube structures formed. Co-culture of platelets and HUVECs at ratios of 1:1.5-25 (HUVECs: platelets) revealed a progressive increase in HUVEC tube formation in the presence of increasing numbers of platelets (Pipili-Synetos, Papadimitriou & Maragoudakis 1998). *In vivo* studies using the aortic ring angiogenesis model also revealed a dose dependent increase in vessel growth in response to platelets (Brill, Elinav & Varon 2004).

In addition to a promotive role, platelets also have the capacity to inhibit angiogenesis, depending on the stimulus. Indeed, platelets can differentially release proteins that

promote or inhibit angiogenesis, through PAR-1 or PAR-4 receptor activation, respectively (Italiano et al. 2008). Further studies revealed that supernatant from ADP-stimulated platelets promoted angiogenesis by HUVECs *in vitro*, whilst inhibition of angiogenesis was observed with released contents from thromboxane A2 (TXA_2)-stimulated platelets (Battinelli, Markens & Italiano 2011). In addition to regulating angiogenesis, platelets also provide support to the vasculature, as demonstrated using the *in vivo* cornea assay, wherein induction of thrombocytopenia resulted in lower vessel numbers and increased haemorrhage in mice (Kisucka et al. 2006).

3.1.2 **Tumour angiogenesis**

Early studies postulated that platelets promote tumour angiogenesis by coming into contact with the tumour microenvironment leading to adherence, activation and subsequent release of potent angiogenesis stimulators such as VEGF (Pinedo et al. 1998; Sabrkhany, Griffioen & Oude Egbrink 2011; Verheul et al. 1997). Using immunofluorescence, platelets are indeed identified in close association with tumour cells in human breast cancer, colorectal cancer, gastric cancer, and hepatocellular cancer (Qi et al. 2015). Platelets have also been observed in ovarian cancer, wherein platelet marker CD41 co-localised with endothelial marker CD31 as well as VEGF (Yuan & Liu 2015). This indicates that platelets can enter the tumour microenvironment and release angiogenic factors such as VEGF (Gonzalez et al. 2004; Verheul et al. 2000a). The elevated level of VEGF in tumour aspirates, in comparison to normal plasma ranges, suggests that tumour angiogenesis may be promoted in cancer patients (Verheul et al. 2000a). Platelet lysates from lung cancer patients have also shown greater potential to induce tube formation by HUVECs *in vitro* in comparison to platelet lysates from healthy controls (Yao et al. 2014).

In vivo experiments also support analyses using patient tissues (Qi et al. 2015; Yuan & Liu 2015), wherein platelet infiltrates are identified in the tumour microenvironment in models of ovarian cancer and in Rip1-Tag2 mice, a mouse model which spontaneously develops pancreatic islet insuloma (Qi et al. 2015; Stone et al. 2012). Using *in vivo* cancer models, a defect in platelet function (P-selectin null mice) resulted in reduced number of infiltrating platelets, reduction in tumour microvessel density and the number of angiogenic islets, reduced VEGF levels in insuloma tissue lysates, and reduced tumour volume in comparison to mice with normal platelet function (Qi et al. 2015). Platelet depletion also impairs tumour angiogenesis in insuloma and ovarian cancer mouse models in comparison to controls (Ringvall et al. 2011; Stone et al. 2012). In the B16/F10 melanoma mouse model, a decrease in the levels of circulating VEGF was associated with impaired tumour angiogenesis following platelet depletion (Qi et al. 2014).

In vivo studies also highlight the importance of platelets in supporting the tumour vasculature. Platelet depletion (using anti-GPIba antibody) led to destabilisation of tumour vessels which resulted in intratumoural haemorrhage in models of Lewis lung carcinoma and B16/F10 mouse melanoma. Transfusion of non-activated (resting) platelets but not degranulated platelets was able to prevent haemorrhage in tumour vasculature (Ho-Tin-Noe et al. 2008).

Collectively, analysis of patient tissues as well as *in vivo* studies reveal a strong importance of platelets in tumour biology, including tumour angiogenesis, growth, and provision of support to the tumour vessels. Whether platelets participate in other pathways that tumours employ for obtaining access to the vasculature, such as vasculogenic mimicry, is unknown.

3.1.3 **Vasculogenic mimicry**

Vasculogenic mimicry (VM), a phenomenon first described by Maniotis and colleagues (1999), is a process wherein aggressive tumour cells can obtain access to the blood supply by secreting their own basement membrane and organising into patterned vessel-like structures lined solely by tumour cells. In addition to aggressive melanoma, VM has also been identified in breast cancer as well as several other cancers (Hess et al. 2007; Maniotis et al. 1999; Shirakawa et al. 2002b). Consistently, patients with VM-positive tumours have significantly poorer survival in comparison to those with VM-negative tumours (Cao et al. 2013; Maniotis et al. 1999; Yang et al. 2016).

The VM patterned structures anastomose with endothelial-lined tumour vasculature or obtain fluid from leaky vessels to actively perfuse the tumour (Hendrix et al. 2003a; Ruf et al. 2003; Shirakawa et al. 2002a). This suggests that platelets can enter the tumour circulation and encounter VM structures in tumours, since red blood cells and plasma have been identified within these structures in a xenograft model of inflammatory breast cancer (Shirakawa et al. 2003). Furthermore, platelets may directly interact with tumour cells that line VM structures as these express various cell-cell adhesion molecules which allow for platelet-tumour cell adhesion (discussed in detail in Chapter 1) (Amo et al. 2014; Bittner et al. 2000; Hendrix et al. 2003a; Trikha et al. 2002).

As discussed above (and in Chapter 1: section 1.4.2.2), VEGF is an important factor in the formation of endothelial-lined tumour vessels and recent studies suggest that the VEGF signalling pathway may also be important in VM. Receptors of VEGF (VEGFR1, VEGFR2 and Neuropilin-1) are expressed by tumour cells capable of VM formation (Misra, Bajaj & Kale 2012; Vartanian et al. 2011a; Yao et al. 2013). Furthermore, VEGF has been identified as an important promotive factor in the formation of VM by human

melanoma cells, ovarian cancer cells, and glioblastoma cells *in vitro* (Francescone et al. 2012; Vartanian et al. 2011a; Vartanian et al. 2007; Wang et al. 2008; Yao et al. 2013). As platelets are a major source of VEGF, these studies suggest that platelets may also participate in VM formation by tumour cells.

In this chapter, the role of platelets in VM is investigated in detail. In light of the current evidence described above it is hypothesised that platelets will mediate an overall promotive effect on VM tube formation by tumour cells *in vitro*. A modified angiogenesis tube formation assay is used to assess VM by tumour cells *in vitro* (Francescone, Faibish & Shao 2011). For these studies, two metastatic melanoma (C32 and CHL-1) and two triple negative breast cancer (TNBC) (MDA-MB-231 and HS-578T) cell lines have been selected (detailed in section 2.3.2) as these are known to develop VM on BME matrix (Sun et al. 2017; Tan et al. 2016).

3.2 **Materials and method**

3.2.1 **Platelet purification protocol**

Blood was collected, using acid citrate dextrose (ACD) anticoagulant, from healthy donors that were between the ages of 18 and 65 years, non-smokers and aspirin/NSAIDs free for at least 1 month. Platelets were purified as described elsewhere using a centrifugation and washing method (Cazenave et al. 2004; Mustard, Kinlough-Rathbone & Packham 1989; Mustard et al. 1972) (also see section 2.3.3). To validate the procedure, the expression of CD42b (platelet specific marker) and CD62P (platelet activation), were assessed by flow cytometry (also detailed in section 2.3.3).

3.2.2 **Tube formation assay**

The capacity for cells (HUVECs or cancer cells) to form tubes was assessed using a tube formation assay previously described (Francescone, Faibish & Shao 2011; Pipili-Synetos, Papadimitriou & Maragoudakis 1998) (see also Chapter 2; section 2.3.5). Growth factor reduced basement membrane extract Geltrex (ThermoFisher Scientific) which contains laminin, collagen IV, entactin, and heparin sulfate proteoglycan, was used in the assay.

Primary HUVECs were seeded at a density of $1x10^4$ cells per well (Moldenhauer et al. 2015). Optimal seeding density is the concentration of cells which result in highest number of tube structures without the formation of large areas of clustered undifferentiated cells. Tumour cells were plated at either optimal seeding density or sub-optimal density (the lowest concentration of cells wherein tube structures were still formed), detailed in section 2.3.5. Based on previous studies (Pipili-Synetos, Papadimitriou & Maragoudakis 1998), co-culture experiments were set up by incubating platelets with either HUVECs or cancer cells at ratios of 1:2, 1:5, 1:10, 1:20, or 1:40

(cells: platelets). Where indicated, platelets were substituted by platelet-sized beads (1-2 µm). After 5 hours (cancer cells) or 8 hours (HUVECs) of incubation, images of tube formation were captured using an Olympus CKX-41 Inverted Microscope (Tokyo, Japan) with 4x objective, fitted with a DP70 digital camera and operated through the Olympus Image Analysis Cell B software program (Olympus, Tokyo, Japan). Tube formation was quantified by counting number of tube-like structures per well (angiogenesis slide) as identified by Image J software (National Institute of Health, USA). Tube-like structures were defined as elongated arrangements of cells that are tightly aligned and extend between collections of cells.

3.2.3 **Statistics**

Values were normalised to the buffer control (HUVEC tube formation or cancer cell VM in the absence of platelets) and one-way analysis of variance (ANOVA) was performed.

3.3 Results

3.3.1 Isolation and optimisation of platelets for culture

To assess the effect of platelets on HUVEC angiogenic potential and compare to previous studies (Pipili-Synetos, Papadimitriou & Maragoudakis 1998), co-culture studies were performed using the angiogenesis tube formation assay. Platelets were collected and purified from healthy donors using an isolation method involving a series of centrifugation and washing steps, and the phenotype was validated using flow cytometry. Purified platelet populations were gated using forward scatter and side scatter profile (Figure 3-1; **A**) and subsequently investigated for expression of the platelet cell marker CD42b (Figure 3-1; **B**) as well as the platelet activation marker CD62P (Figure 3-1; **C**). Analysis of the purified human platelet population revealed high purity and a low level of baseline activation, as indicated by uniform expression of CD42b and minimal peak shift of CD62P expression in comparison to isotype control (Figure 3-1; **C**). Mean positive expression was 90.6% and 13.5%, respectively (Figure 3-1; **D**).

HUVECs were cultured in the presence or absence of platelets using an optimal seeding density. Co-culture with platelets at the indicated ratios had no effect on HUVEC tube formation in comparison to buffer control (Figure 3-2; **A-B)**. In previous studies wherein platelets promote tube formation, HUVECs were cultured at low concentrations (sub-optimal; 20,000 cells/cm^2 versus the optimal 80,000 cells/cm^2 in the current study) (Pipili-Synetos, Papadimitriou & Maragoudakis 1998). It was thus hypothesised that the current seeding density may not allow for further increases in tube formation. To test this, a sub-optimal seeding density was established for the assay (Figure 3-2; **C)**. As shown in Figure 3-2 (**D)**, no difference in tube formation at sub-optimal seeding density was observed in the presence of platelets when compared to buffer control.

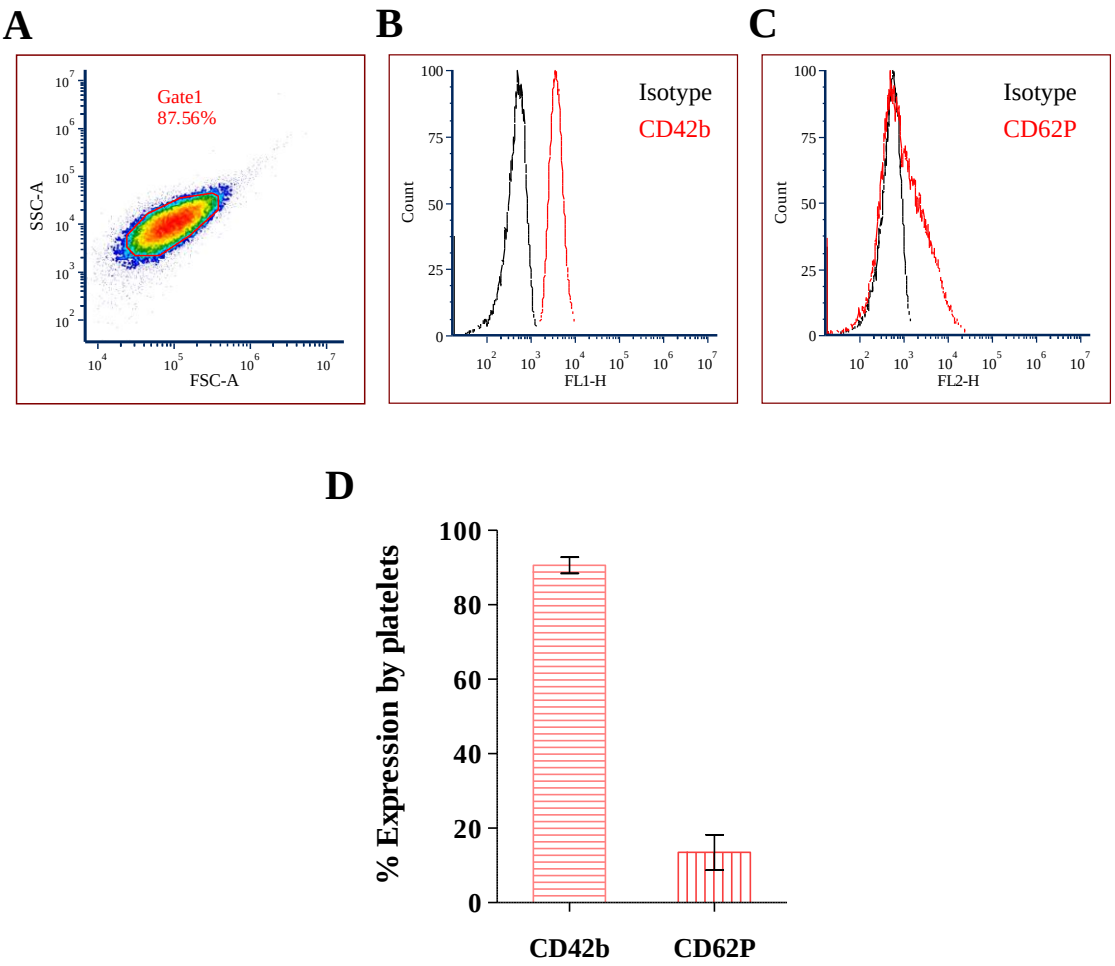


Figure 3-1. Validation of platelet isolation and purification

Platelets purified from healthy donors were analysed using flow cytometry. (**A**); Representative flow cytometry event plots showing gated platelet population. (**B**) The gated population was stained for expression of CD42b and (**C**) CD62P. (**D**) Percent expression of platelets stained positive was quantified and data are expressed as mean ± SEM. Results are pooled from 7 separate experiments (different donors).

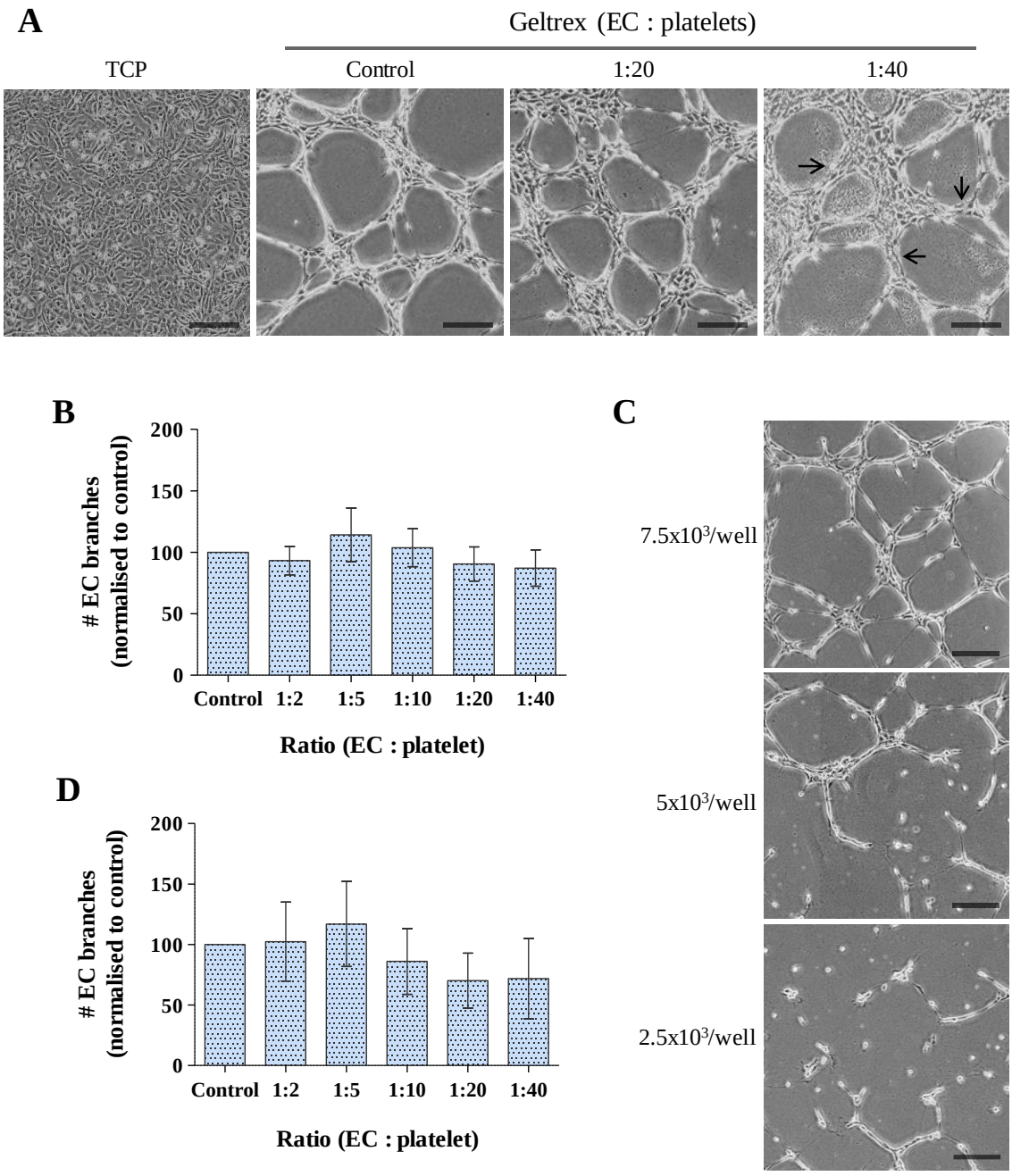


Figure 3-2. The angiogenic capacity of HUVECs in the presence of platelets *in vitro*

(**A**) Representative images of HUVECs cultured on tissue culture plastic (TCP) in the absence of platelets, or on Geltrex either alone (control) or with platelets at the indicated ratio (EC: platelets). Arrows indicate platelets in association with tube structures. (**B**) Quantification of vascular-like structures using optimal seeding concentration of HUVECs ($1x10^4$/well). (**C**) Three HUVEC concentrations were tested to find sub-optimal seeding concentration. (**D**) Sub-optimal seeding concentration of HUVECs ($5x10^3$/well) cultured with or without platelets. In (B) and (C), there was no significant difference between the groups as determined by one-way ANOVA. Data are expressed as mean ± SEM. Results are pooled from 5 separate experiments (different donors). Scale bar is 200µm, original magnification (**A**) 40x, and (**C**) 100x.

3.3.2 The effect of platelets on VM formation by melanoma cell lines

To determine whether platelets would promote VM formation as hypothesised, VM formation by human metastatic melanoma cell lines was assessed using the assay described above. This was performed using the optimal cell seeding concentrations for VM formation by two human melanoma cell lines, C32 ($2x10^4$/well) and CHL-1 ($1.5x10^4$/well). In response to platelets, VM formation by C32 melanoma cells was significantly inhibited compared to the buffer control. This effect was observed at seeding ratios of 1:20 and 1:40 (cell: platelets) (Figure 3-3; **A** and **C**). Notably, the higher platelet seeding ratios (1:20, and 1:40) affected the morphology of the VM structures formed whereby these structures were thinner (comprised of fewer cells) in the presence of high platelet-to-cancer cell ratio in comparison to VM structures formed in buffer control. At high magnification (insert), platelets were observed on the surface of rounded C32 melanoma cells at high platelet-to-cancer cell ratio, and platelet-cancer cell aggregates were consistently observed. Although a similar trend was observed, the presence of platelets had no statistically significant effect on VM by CHL-1 melanoma cells in comparison to buffer control (Figure 3-3; **B** and **D**). Platelets were observed in close association with VM formed structures by CHL-1 melanoma cells at high platelet-to-cancer cell ratio, with few platelet-cancer cell aggregates observed (high magnification insert).

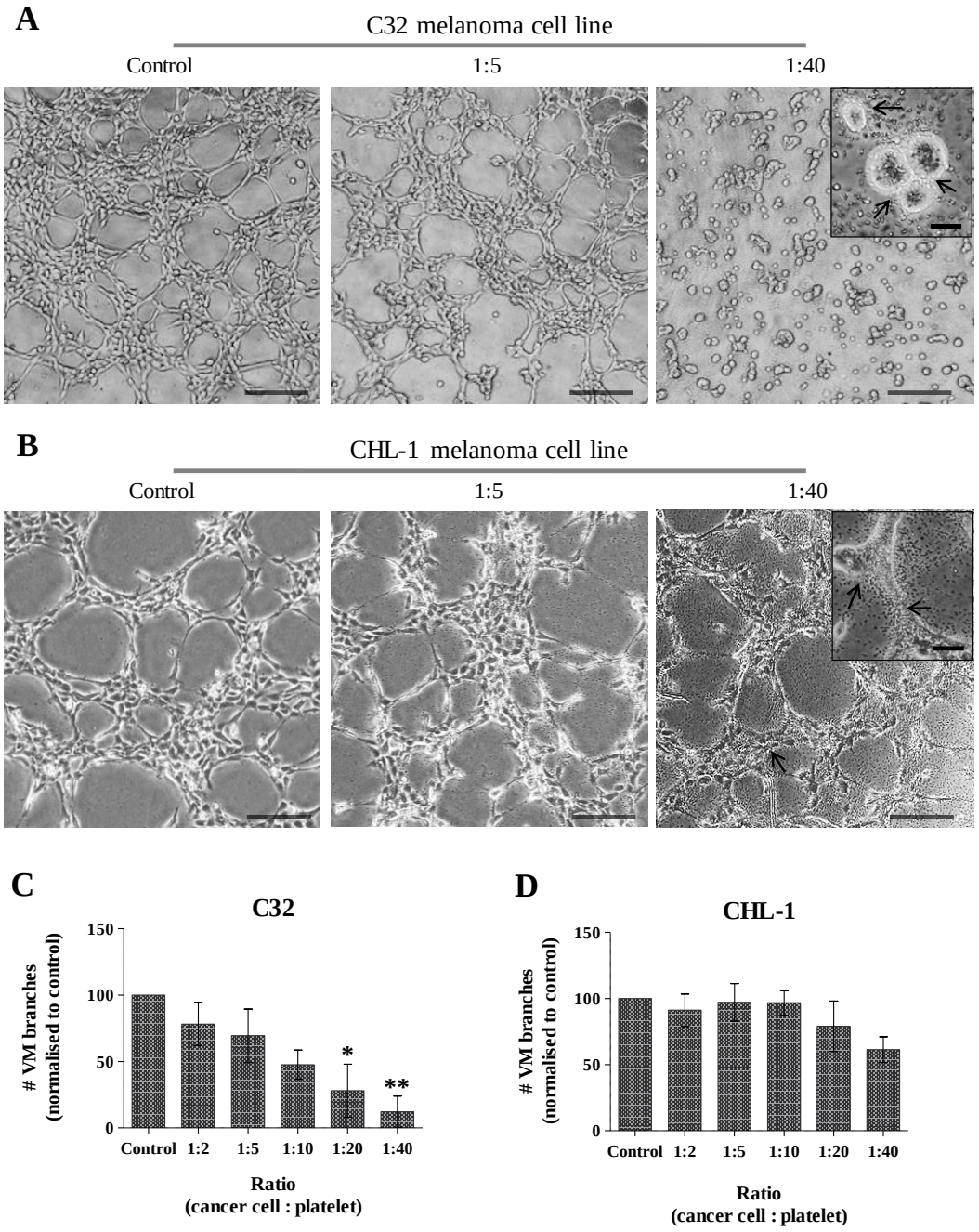


Figure 3-3. Platelets selectively inhibit VM formation by melanoma cell lines *in vitro*

(**A-B**) Representative images showing C32 melanoma cells (A) and CHL-1 melanoma cells (B) treated with buffer control or platelets at the indicated ratio (cells: platelets). (**C-D**) VM vessel formation by C32 and CHL-1 melanoma cells was quantified following treatment with increasing platelet concentration. Black arrows indicate platelet-cancer cell aggregates and platelet contact with tumour cells in VM structures. One-way ANOVA, data are expressed as mean ± SEM. Results are pooled from 3 separate experiments (different donors). **P*<0.05, ***P*<0.01, one-way ANOVA. Scale bar is 200µm (40 µm for insert), original magnification 40x (400x for insert).

3.3.3 The effect of platelets on VM formation by breast cancer cell lines

The assay described above was also used to assess VM formation by breast cancer cell lines. Platelets were co-cultured with cancer cells at the indicated ratios using optimal cell seeding concentrations of cancer cell lines, HS-578T and MDA-MB-231 ($1.5x10^4$/well).

The presence of a high platelet-to-cancer cell ratio was found to inhibit VM formation by breast cancer cells in comparison to buffer control (Figure 3-4; **A-B**). The inhibitory effect of platelets on VM formation was statistically significant in both breast cancer cell lines at the highest platelet-to-cancer cell ratio (Figure 3-4; **C-D**). Although similar to effects observed in platelet and C32 melanoma cell co-culture, this effect was less pronounced in MDA-MB-231 and HS-578T breast cancer cell lines.

The VM structures formed by MDAMB-231 and HS-578T breast cancer cell lines were thinner (comprised of fewer cells) in the presence of high platelet-to-cancer cell ratio in comparison to VM structures formed in buffer control. High magnification (inserts) images indicated that platelets interacted closely with breast cancer cells. In the presence of a high platelet-to-cancer cell ratio, breast cancer MDA-MB-231 cells were more rounded, morphologically, in comparison to a low platelet-to-cancer cell ratio or buffer control.

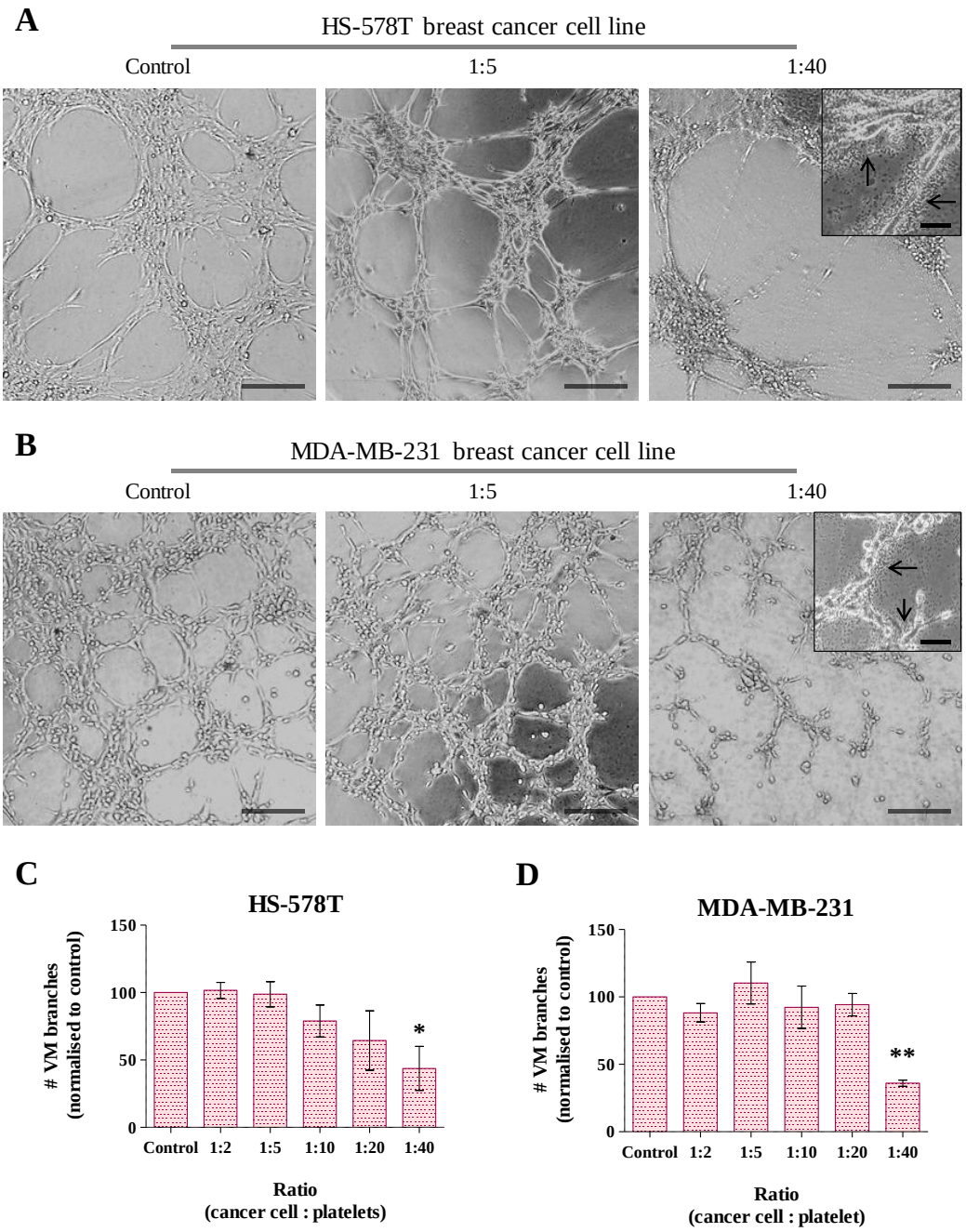


Figure 3-4. Platelets inhibit VM formation by breast cancer cell lines *in vitro*.

(**A-B**) Representative images showing HS-578T breast cancer cells (A) and MDA-MB-231 breast cancer cells (B) treated with buffer control or platelets at the indicated ratio (cells: platelets). (**C-D**) VM vessel formation by HS-578T and MDA-MB-231 breast cancer cells was quantified following treatment with increasing platelet concentration. Black arrows indicate platelet contact with tumour cells in VM structures. One-way ANOVA, data are expressed as mean ± SEM. Results are pooled from 3 separate experiments (different donors). **P*<0.05, ***P*<0.01, one-way ANOVA. Scale bar is 200µm (40 µm for insert), original magnification 40x (400x for insert).

3.3.4 The effect of platelets on VM using sub-optimal cancer cell seeding density

To determine whether using an optimal seeding concentration of cancer cells can potentially mask any increases in VM due to unavailability of space in the assay, experiments using sub-optimal cancer cell seeding concentrations were performed. Based on previous experiments, the VM competent C32 melanoma cells, which were most sensitive to the anti-VM effect of platelets, and a breast cancer cell line, HS-578T were used. As shown in Figure 3-5, C32 melanoma cells demonstrated VM formation at the sub-optimal seeding concentration of $1.5x10^4$ cells/well (the lowest concentration of cells wherein tube structures still formed) (Figure 3-5; **A**), while HS-578T breast cancer developed VM at a low seeding concentration of $7.5x10^3$ cells/well (Figure 3-5; **B**).

Experiments to test the effect on VM formation using sub-optimal cancer cell concentrations in the presence of platelets were performed in the same manner as described with optimal cell seeding concentrations. Co-culture experiments were performed using in the same platelet-to-cancer cell ratios as previously described. The presence of platelets appeared to significantly inhibit the formation of VM structures by both C32 melanoma (Figure 3-6; **A**) and HS-578T breast cancer cells (Figure 3-6; **B**), in comparison to buffer control (Figure 3-6; **C**-**D**). The platelet inhibitory effect was greater using sub-optimal cancer cell concentrations in comparison to that observed using optimal cancer cell seeding concentrations. Notably, the presence of platelets produced a more prominent inhibitory effect on VM in the C32 melanoma cell line compared to the HS-578T breast cancer cell line.

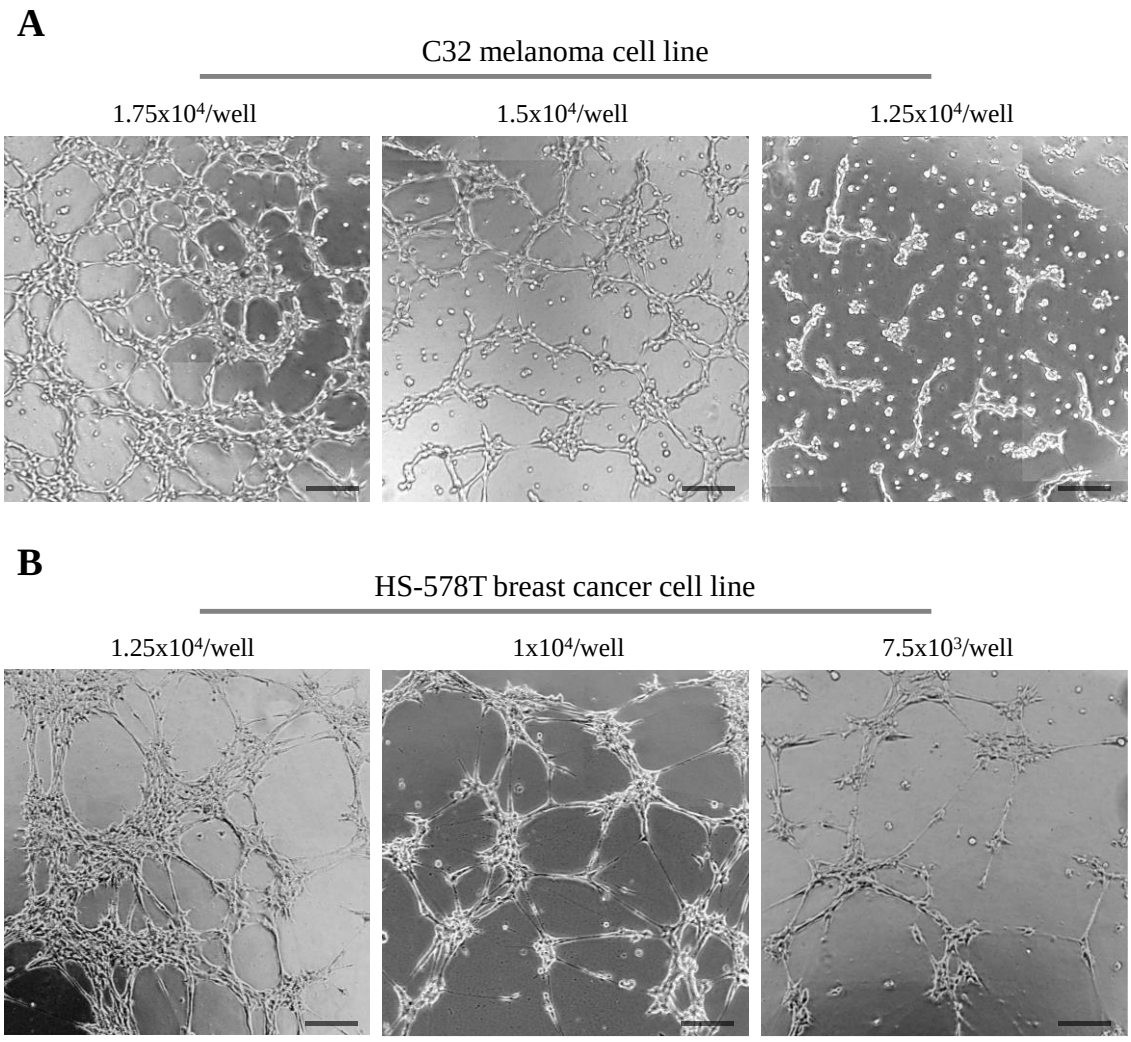


Figure 3-5. Sub-optimal cancer cell concentration

(**A-B**) VM formation was tested using three concentrations of (**A**) C32 melanoma cells and (**B**) HS-758T breast cancer cells to find sub-optimal tube formation (C32; $1.5x10^4$/well and HS-578T; $7.5x10^3$/well). Scale bar is 200µm, original magnification 100x.

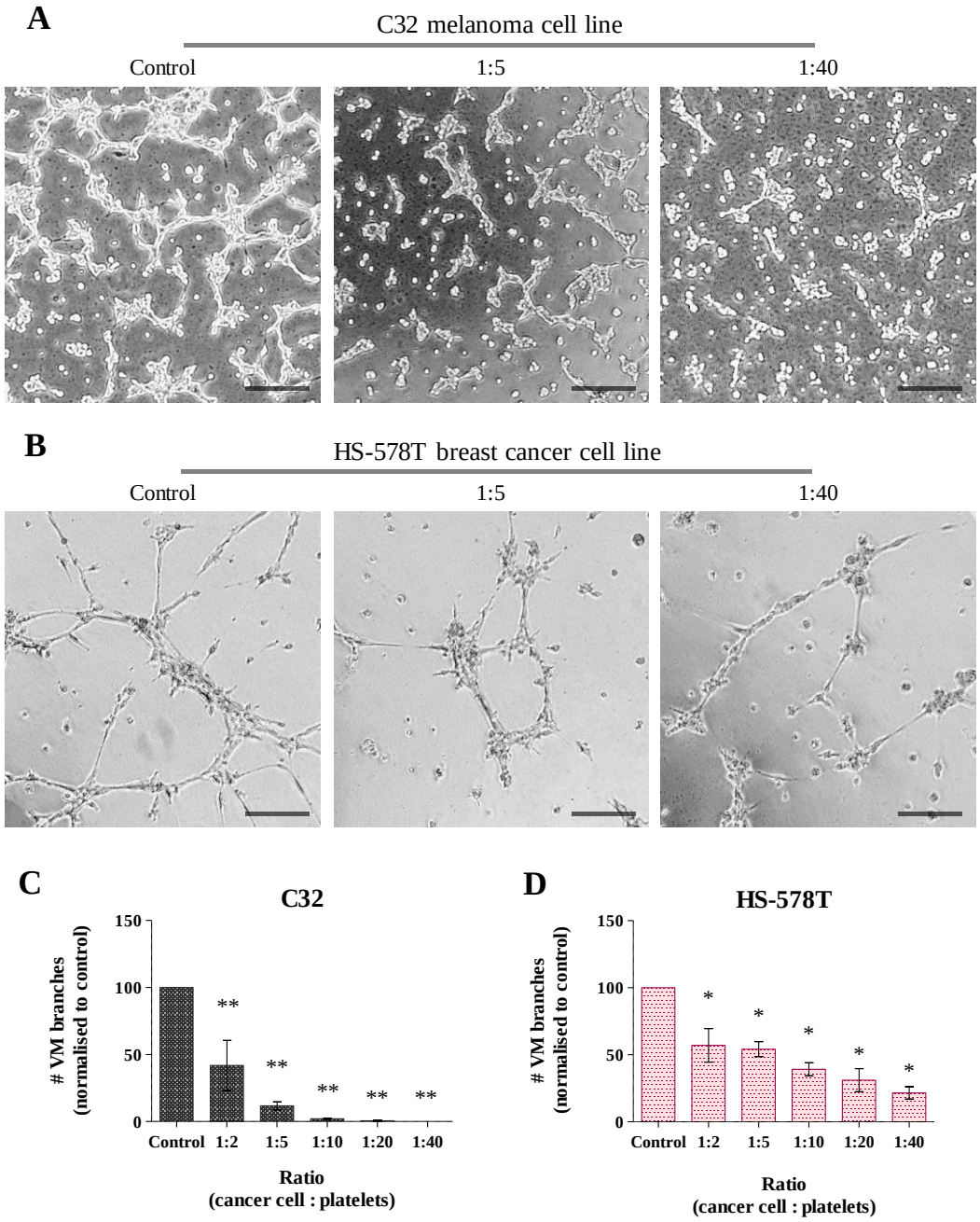


Figure 3-6. Sub-optimal cancer cell seeding concentration exaggerates the platelet effect on VM *in vitro*.

In (**A**); representative images showing C32 melanoma cells and in (**B**) HS-578T breast cancer cells at sub-optimal VM-forming seeding concentrations, treated with buffer control or platelets at the indicated ratio (cell: platelets). Sub-optimal concentrations were $1.5x10^4$ cells/well for C32 melanoma cell line, and $7.5x10^3$ cells/well for HS-578T. (**C**) VM vessel formation by cancer cells C32 and HS-578T was quantified and data are expressed as mean ± SEM. Results are pooled from 3 separate experiments (different donors). **P*<0.01, ***P*<0.001, one-way ANOVA. Scale bar is 200µm, original magnification 40x.

3.3.5 **Mechanism of platelet action**

To confirm that the effect observed in cancer cell VM was not mediated simply through spatial interference but through an active platelet-specific process, inert platelet-sized (i.e. 2µm) polystyrene microspheres were added to the VM assay in place of platelets. As shown in Figure 3-7, platelet-sized beads were co-cultured with at a ratio of 1:40 (cell: beads) with HS-578T breast cancer (Figure 3-7; **A**) and C32 melanoma (Figure 3-7; **B**).

The VM by HS-578T breast cancer cell line was not affected by the presence of platelet-sized beads at the ratio 1:40 (cell: beads ratio) (Figure 3-7; **C**) whereas the presence of platelets at the same ratio was inhibitory. This indicates that the inhibition is not due to a spatial interference.

While both ratios (1:20 and 1:40) of platelet-sized beads produced a small downward trend in tube formation by the C32 melanoma cell line, only the 1:40 ratio significantly inhibited VM formation. Notably, the magnitude of this inhibition was much less using platelet-sized beads compared to platelets. Since platelets inhibit VM by C32 melanoma cells at both 1:20 and 1:40, this indicates that the inhibition at 1:20 ratio is platelet specific and not due to spatial interference while at the 1:40 ratio may be partially caused by spacial interference but also due to a specific effect (Figure 3-7; **D**). The platelet inhibiting effect of VM on HS-578T breast cancer cells (at 1:40 ratio) and C32 melanoma cells (1:20 ratio) is platelet-specific.

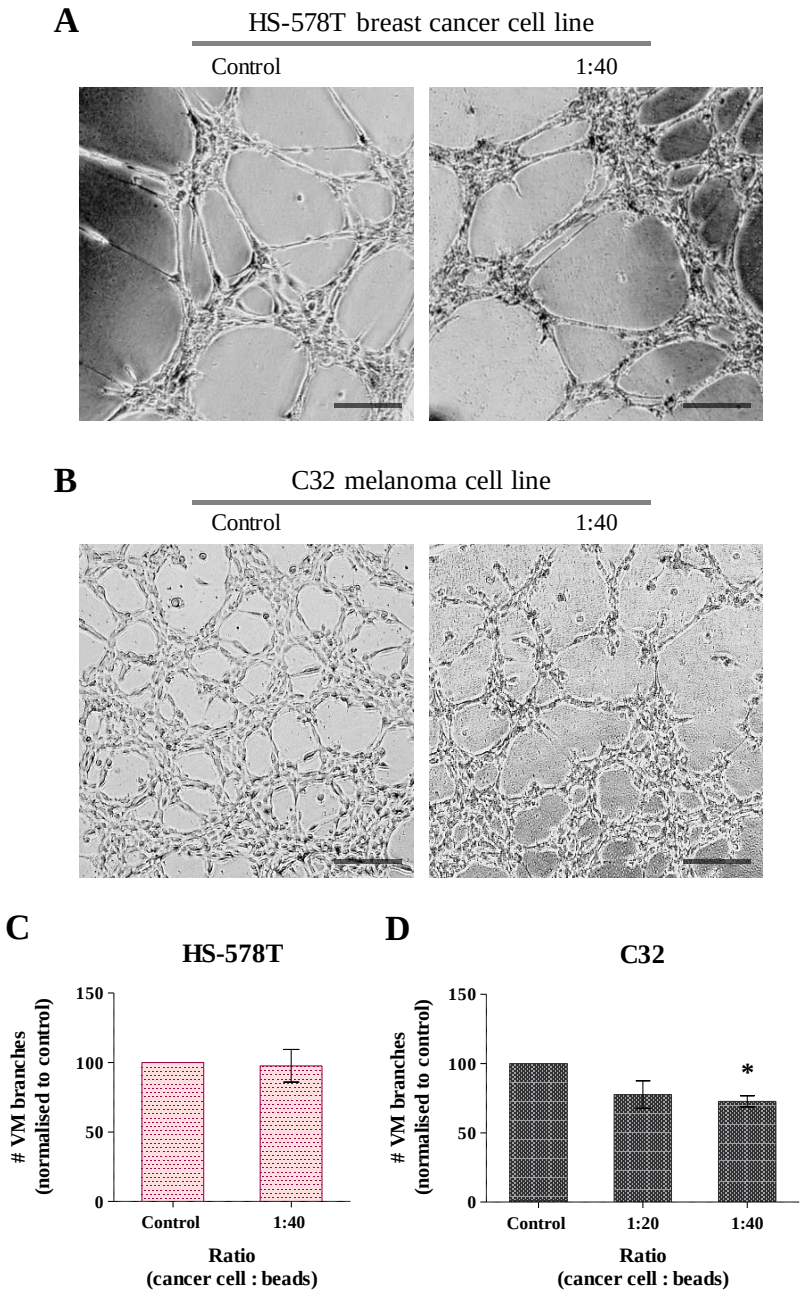


Figure 3-7. VM formation by cancer cells in the presence of platelet-sized beads.

In (**A**); representative images showing HS-578T breast cancer cells and in (**B**); C32 melanoma cells treated with buffer control or platelet-sized beads (2um) at the indicated ratio (cells: beads). In (**C**); VM vessel-like structure formation by cancer cells HS-578T and C32 in (**D**) in the presence of platelet-sized beads was quantified and data are expressed as mean ± SEM. Results are an average of 3 separate experiments. *$p < 0.05$ compared with buffer control, one-way ANOVA. Scale bar is 200µm, original magnification 40x.

3.4 **Discussion**

Evidence in the literature suggests that platelets can enter a tumour and come into contact with VM channels (Ruf et al. 2003; Shirakawa et al. 2003; Shirakawa et al. 2002b). In this chapter, the effect of platelets in VM formation by breast cancer and melanoma cancer cell lines was assessed by co-culture studies *in vitro* using a modification of the angiogenesis tube formation assay. This led to the identification of a previously unknown role of platelets in regulating the formation of VM channels by highly malignant cancer cell lines *in vitro*.

Platelets were isolated and purified using a series of centrifugation and washing steps (Cazenave et al. 2004; Mustard, Kinlough-Rathbone & Packham 1989; Mustard et al. 1972). Staining with CD42b (platelet specific marker, glycoprotein Ibα) and CD62P (marker of platelet activation, P-selectin) and analysis by flow cytometry indicated that the isolation protocol resulted in a highly pure population of platelets with low levels of activation. The latter indicates that secretion of granule contents is minimal (Lu & Malinauskas 2011). This is ideal as platelet activation during the isolation procedure should be avoided to ensure that platelets retain their normal secretory function for *in vitro* studies (Cazenave et al. 2004; Mustard, Kinlough-Rathbone & Packham 1989).

Studies in this chapter were based on the work by Pipili-Synetos and colleagues who were the first to demonstrate that platelets promote angiogenesis by HUVECs *in vitro* (Pipili-Synetos, Papadimitriou & Maragoudakis 1998). To recapitulate the results of the above study, the effect of platelets was tested on tube formation by primary HUVECs. In contrast to previous published data, the current studies have found that at low or high platelet-to-HUVEC ratios, platelets did not promote angiogenesis in comparison to buffer control. It was hypothesised that the optimal seeding concentration used in the present

study may have masked platelet-induced increases in tube formation. Therefore, tube formation at a sub-optimal HUVEC seeding concentration was tested as well, which also showed no differences in comparison to buffer control. Although platelets did not alter the extent of tube formation observed using either optimal or sub-optimal HUVEC seeding concentrations, high magnification images indicated that platelets appeared to closely interact with tube-like structures formed by HUVECs. This suggested that platelets can actively interact within the system and this is consistent with studies by Pipili-Synetos and colleagues (1998). The interaction between platelets and HUVECs is mediated through several cell surface molecules including P-selectin and GPIIb/IIIa on platelets with PSGL-1 and integrin $\alpha_v\beta_3$ on endothelial cells, respectively (van Gils, Zwaginga & Hordijk 2009).

In addition to angiogenesis promoters, it is well known that platelets also contain angiogenesis inhibitors which have inhibitory effects on tumour angiogenesis (Good et al. 1990; O'Reilly et al. 1997; O'Reilly et al. 1994). Others have demonstrated that angiogenic promoters and inhibitors are packaged into distinct alpha granules, and these can be differentially released depending on the stimulus (Italiano et al. 2008). Platelet stimulation with ADP induced VEGF release (angiogenesis promoter) whereas TXA_2 stimulation induced endostatin release (angiogenesis inhibitor). The data obtained in the current study indicated that platelets can interact with HUVECs, and may release a balance of both angiogenic promoters and inhibitors ultimately resulting in no overall effect on tube formation by HUVECs *in vitro.* Evidently, the current result differs from previously published data by Pipili-Synetos and colleagues, and this disparity may be due to variations in experimental methods between the studies (Pipili-Synetos, Papadimitriou & Maragoudakis 1998). Notably, the 1998 study isolated platelets in the presence of aspirin, which has been shown to decrease platelet-released contents including levels of

thrombospondin and platelet-factor 4, which both have potent anti-angiogenic potential (Coppinger et al. 2007). However, Pipili-Synetos and colleagues showed that aspirin did not affect the ability of platelets to promote HUVEC tube formation. This is in direct contrast to studies by Battinelli and colleagues which found that aspirin inhibited platelet-mediated tube formation by HUVECs (Battinelli, Markens & Italiano 2011). Additional studies are necessary to further understand the role of platelets in tube formation by HUVECs and reconcile the disparity in results.

The current studies suggested that platelets may release a balance of pro- and anti-angiogenic compounds which result in no overall effect on HUVEC tube formation. To determine whether this balance is maintained during the formation of VM channels, VM formation by highly malignant cancer cells was assessed in the presence of platelets. Platelets inhibited VM formation by cancer cell lines (C32 melanoma, MDA-MB-231 and HS578T breast cancer) at the highest platelet-to-cancer cell ratio (1:40). In the presence of platelets (at the same 1:40 ratio), VM formation was unaffected in one cancer cell line (CHL-1 melanoma). The use of low cancer cell seeding concentrations confirmed that optimal VM forming concentrations did not mask any increase in VM formation, and in fact amplified the platelet-inhibitory effect on VM formation by malignant melanoma (C32) and breast cancer (HS-578T) cell lines.

Platelets were identified on the surface of cancer cells and platelet-cancer cell aggregates were also observed (C32 melanoma cell line). In addition, platelets appeared in close association with the cancer cells in the VM structures *in vitro*. The formation of platelet-cancer cell aggregates in culture has been described by others, a phenomenon known as tumour cell-induced platelet aggregation (TCIPA) (Heinmoller et al. 1996; Jurasz, Alonso-Escolano & Radomski 2004; Lindenmeyer, Legrand & Menashi 1997). Others have demonstrated that both melanoma and breast cancer cells induce TCIPA (Chang et

al. 2015; Qi et al. 2014). The formation of these aggregates can occur since platelets and tumour cells interact through various surface receptors including integrin $\alpha_v\beta_3$ (with platelet GPIIb/IIIa) and PSGL-1 (with platelet P-selectin) (Amo et al. 2014; Bittner et al. 2000; Gong et al. 2012a; Hendrix et al. 2003a; Trikha et al. 2002). In addition, laminin is also abundantly expressed by VM channels formed by malignant melanoma cells *in vitro* (Hendrix et al. 2003a). Platelets can interact with laminin through integrin $\alpha_6\beta_1$ and collagen receptor GPVI (Ozaki, Suzuki-Inoue & Inoue 2009). These data suggest that platelets interact with VM channels *in vitro* through various molecules but that the platelet inhibitory effect on VM formation may depend on the cancer phenotype since not all cancer cell lines respond in the same manner.

To investigate the specificity of the platelet-inhibitory effect on VM formation, platelets were replaced with inert platelet-sized (2µm) polystyrene microspheres. While no effect was observed on VM by HS-578T breast cancer cells, a significant inhibitory effect on VM by C32 melanoma cells was observed at the cell: beads ratio of 1:40. Notably, this effect was smaller in magnitude when compared with platelets at the same ratio, indicating that platelets inhibit VM by C32 melanoma cells partially through a spacial interference at high concentrations, but also through direct interactions. The study by Pipili-Synetos and colleagues found that platelets promoted angiogenesis by HUVECs through a direct interaction only, and not through released contents from α-thrombin-activated platelets *in vitro* (Pipili-Synetos, Papadimitriou & Maragoudakis 1998). Conversely, more recent studies have shown that contents released from platelets activated by exposure to agonists (ADP or breast cancer cells) can induce angiogenesis by HUVECs *in vitro* (Battinelli, Markens & Italiano 2011; Battinelli et al. 2014). The mechanism behind the inhibitory effect of platelets on VM formation by malignant cancer cell lines will be investigated in the next chapter.

3.1 **Summary**

The studies described in this chapter revealed that platelets inhibit VM formation in melanoma cell line C32, and breast cancer cell lines MDA-MB-231 and HS-578T but had no effect on VM formation by CHL-1 melanoma cells. This effect is not solely due to spatial interference. Investigations designed to elucidate the mechanism through which platelets mediate this effect are the focus of the next chapter.

Chapter 4. Investigating the mechanistic link between platelets and VM by cancer cells

4.1 Introduction

In Chapter 3, the role of platelets in VM formation by malignant cancer cells was investigated using the *in vitro* VM tube formation assay. These results showed that platelets inhibited VM formation by the melanoma cell line C32, as well as the breast cancer cell lines MDA-MB-231 and HS-578T but had no effect on VM formation by the melanoma cell line CHL-1. Chapter 4 extends this work by focusing on the mechanism/s by which platelets attenuate VM formation *in vitro*.

4.1.1 Adhesion molecules mediating interactions between platelets and cancer cells

As shown in Chapter 3, platelets interfere with VM formation by cancer cells via a contact-dependent process. To identify the molecular mechanisms underpinning this process, a better understanding of the adhesion molecules involved is required. As illustrated in Figure 4-1, platelets and cancer cells can interact through various cell surface molecules. The most common interactions are those occurring through (i) activated platelets expressing P-selectin which can bind to P-selectin glycoprotein ligand-1 (PSGL-1) documented to be expressed by cancer cells (Gong et al. 2012a; Gong et al. 2012b; Kim et al. 1998a) and (ii) via glycoprotein GPIIb/IIIa expressed by platelets and integrin $\alpha_v\beta_3$ expressed by cancer cells. More specifically, it is the arginine-glycine-aspartic acid (RGD) binding sequences within these integrins that facilitate cell-cell interactions through RGD containing components such as fibrinogen (Figure 4-1) (Engebraaten et al. 2009; Felding-Habermann et al. 1996; Trikha et al. 2002). It is through these interactions that platelets assist and promote cancer progression (e.g. tumour growth, tumour cell migration and survival in circulation as well as metastasis) (Amo et al. 2014; Gay & Felding-Habermann 2011; Kim et al. 1998a).

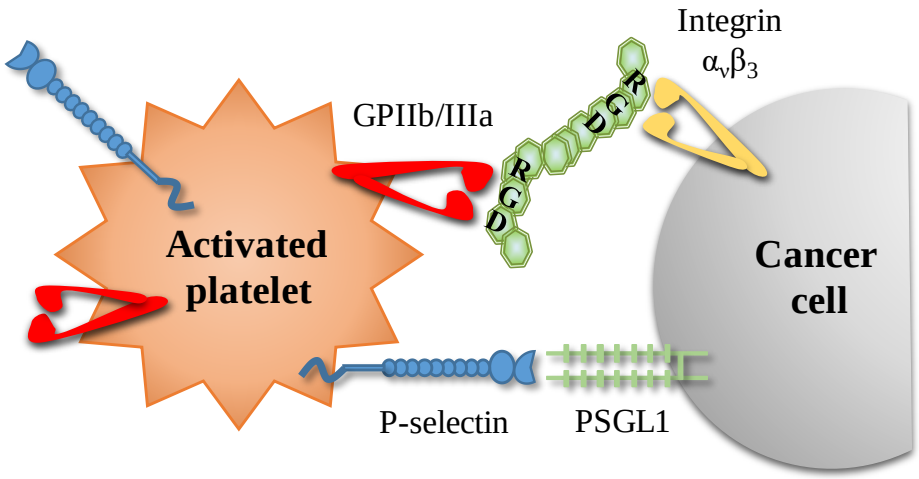


Figure 4-1. Cancer cell interaction with platelets through cell surface proteins

Cancer cells expressing integrin $\alpha_v\beta_3$ can interact with platelets via the RGD sequences of extracellular matrix (ECM) components such as fibrinogen and platelet expression of GPIIb/IIIa. Cancer cells can also directly interact with platelets via their expression of P-selectin glycoprotein ligand-1 (PSGL-1) which binds to the P-selectin expressed by activated platelets.

Gong and colleagues showed that platelets isolated from healthy donors (which express <10% P-selectin when in a resting (inactivated) state) formed few platelet-cancer cell aggregates in co-culture with the human lung carcinoma cell line A549, while platelets isolated from lung cancer patients (identified to be more activated with >50% expression of P-selectin) formed significantly more aggregates (Gong et al. 2012a). This interaction was inhibited via knockdown of PSGL-1 on the cancer cells. Similar results have also been observed *in vivo* wherein aggregate formation between platelets and cancer cells was attenuated in P-selectin knockout mice (Borsig et al. 2001; Kim et al. 1998a). In a mouse model of melanoma, P-selectin deficiency also inhibited tumour growth and metastasis (Qi et al. 2015; Qi et al. 2014). Similarly, colon carcinoma growth and metastasis was also reduced in P-selectin knockout mice (Kim et al. 1998a).

As illustrated in Figure 4-1, cancer cells expressing $\alpha_v\beta_3$ integrin adhere, via RGD containing compounds, to platelets expressing GPIIb/IIIa. Blocking either $\alpha_v\beta_3$ integrin expression on melanoma cells or GPIIb/IIIa expression on platelets was shown to inhibit cancer cell-platelet interactions *in vitro* (Lonsdorf et al. 2012). *In vivo* studies also documented the importance of platelet-expressed GPIIb/IIIa and cancer cell-expressed integrin $\alpha_v\beta_3$ in xenograft models of human melanoma in mice and rats (Trikha et al. 2002). Blocking of melanoma cell-expressed $\alpha_v\beta_3$ partially inhibited tumour growth. However, blocking a combination of platelet expressed GPIIb/IIIa, EC-expressed $\alpha_v\beta_3$ and tumour cell-expressed $\alpha_v\beta_3$ via blocking antibody administered five times/week significantly attenuated tumour formation in nude rats. Blocking the α_v subunit of $\alpha_v\beta_3$ integrin is also sufficient to inhibit metastasis in a mouse model of melanoma (Lonsdorf et al. 2012). On the other hand, Pang and colleagues showed that blocking antibodies to platelet expressed GPIIb/IIIa and P-selectin could attenuate the metastatic capacity of MDA-MB-231 breast cancer cells cultured with washed thrombin-activated platelet

membranes (Kut, Mac Gabhann & Popel 2007). Collectively, these studies suggest that GPIIb/IIIa, $\alpha_v\beta_3$, PSGL-1 and P-selectin are important in mediating cross-talk and contact between platelets and cancer cells.

4.1.2 **Platelet activation and factor release**

In addition to directly interacting with platelets, cancer cells can also secrete platelet activating factors, such as thrombin (Factor (F) II) (Heinmoller et al. 1995; Heinmoller et al. 1996), and tissue factor (FIII; activating the extrinsic coagulation pathway; Chapter 1, Figure 1-3) (Mueller et al. 1992; Ueno et al. 2000). Tumour cells can generate thrombin through expressing tissue factor (TF) which binds to FVIIa to activate factors IX (to IXa) and X (to Xa) (Chapter 1, Figure 1-8) (Nierodzik & Karpatkin 2006). Activated FX (FXa) initiates the common pathway which leads to the formation of the prothrombinase complex (composed of FXa, FVa), allowing cancer cells to convert prothrombin to thrombin (Kirszberg, Rumjanek & Monteiro 2005; VanDeWater et al. 1985). Studies using IHC demonstrated that tumour cells can express both prothrombin (FII), and tissue factor (FIII) (Wojtukiewicz et al. 2001).

Table 4-1 lists several agents, which are classified as either *weak* or *strong* platelet agonists. Strong agonists induce shape change, aggregation and factor release in platelets, whereas weak agonists do not have the capacity for generating all platelet functional endpoints (Cattaneo 2009; Huang & Detwiler 1981). To date, approximately 300 molecules have been identified as being released by platelets following thrombin activation (summarised in Chapter 1, Table 1-1) including pro-angiogenic factors, anti-angiogenic factors, haemostatic factors, proteases, matrix proteins, necrotic factors, and inflammatory cytokines and chemokines (Coppinger & Maguire 2007; Gear & Camerini 2003; Jurasz et al. 2003; Peterson et al. 2010; Whiteheart 2011).

Table 4-1. Strong and weak platelet agonists

Agonist	Strength of platelet agonist	References
Thrombin	**Strong**	(Chung et al. 2002; Zhou & Schmaier 2005)
Collagen	**Strong**	(Chung et al. 2002; Zhou & Schmaier 2005)
Thromboxane A2	**Strong**	(Cattaneo 2009; Jagroop et al. 2000)
Arachidonic acid	**Strong**	(Huang & Detwiler 1981; Saggu et al. 2013)
ADP	**Weak**	(Cattaneo 2009; Jagroop et al. 2000)
Adrenalin	**Weak**	(Jagroop et al. 2000; Zhou & Schmaier 2005)
Platelet activating Factor	**Weak**	(Rao 2003; Rao et al. 1984)

In response to different stimuli, platelets can release angiogenic factors which either promote or inhibit the local vasculature. For example, Italiano and colleagues demonstrated that substances contained within platelets are organised into different α-granules and that stimulation of platelets via PAR-1 or ADP causes the release of pro-angiogenic proteins whilst activation through PAR-4 or TXA_2 causes the release of anti-angiogenic factors (Battinelli, Markens & Italiano 2011; Italiano et al. 2008). These studies are also supported by the work of others (Chatterjee et al. 2011), and collectively they demonstrate the diversity in effects that platelets can mediate depending on their activation triggers.

In Chapter 3 it was shown that platelets actively inhibit VM formation by various cancer cell lines *in vitro*. Based on current studies in the literature, it is tempting to speculate that the inhibitory effect that platelets have on VM formation by some cancer cell lines may also be a function related to anti-angiogenic factors they contain, such as angiostatin, endostatin, and thrombospondin-1 (TSP-1). Testing this is the aim of this Chapter.

4.1.2.1 ***Angiostatin***

Angiostatin was first discovered and isolated as a 38-kDa protein purified from proteolysis of plasminogen, a fibrinolytic system enzyme (O'Reilly et al. 1994). Plasminogen can be cleaved by a variety of enzymes including MMPs and tissue-type plasminogen activator, which are contained within platelets and released following thrombin activation (Coppinger et al. 2004). Jurasz and colleagues demonstrated that human platelets can generate angiostatin following aggregation with collagen, thrombin, or fibrosarcoma cells (Jurasz et al. 2003). The anti-angiogenic properties of angiostatin were first demonstrated by O'Reilly and colleagues who identified a dose dependent inhibitory effect using the chick embryo model of angiogenesis (O'Reilly et al. 1994). A

blocking antibody against the angiostatin released by platelets has also been found to promote *in vitro* tube formation by HUVECs (Jurasz et al. 2003).

Early studies also revealed that angiostatin attenuates primary tumour growth and growth of secondary metastases in the Lewis lung carcinoma (LLC) mouse model (O'Reilly et al. 1994). This effect was consistent across a variety of xenograft models including breast cancer, colon carcinoma, and prostate cancer (O'Reilly et al. 1996). Indeed, continuous treatment with angiostatin (50 mg/kg every 12 hours) almost completely inhibited tumour growth.

Annexin II, a receptor for plasminogen, was shown to regulate the anti-angiogenic functions of angiostatin (Sharma & Sharma 2007; Tuszynski et al. 2002). The binding of angiostatin to Annexin II inhibits plasminogen activation and thus the formation of plasmin, preventing EC and tumour cell migration and invasion (Stack et al. 1999). Angiostatin can also bind to integrin $\alpha_v\beta_3$ thus acting as an antagonist to inhibit the binding of RGD containing factors (Tarui, Miles & Takada 2001).

4.1.2.2 ***Endostatin***

Endostatin, a 20-kDa C-terminal fragment of collagen XVIII, inhibits EC proliferation, angiogenesis, primary tumour growth and growth of secondary metastases in the LLC mouse model (O'Reilly et al. 1997). Endostatin is released from the α-granules of platelets following thrombin activation of PAR-4 (Ma et al. 2011).

Whilst the signalling mechanisms regulating the effects of endostatin are not fully established, studies show that endostatin regulates the expression of ~12% of the genome in human dermal microvascular ECs, specifically, the upregulation of anti-angiogenic genes and downregulation of pro-angiogenic genes (Abdollahi et al. 2004). It is also

known that endostatin prevents VEGF binding to VEGFR1 and VEGFR2 thus inhibiting VEGF-induced tyrosine kinase phosphorylation of VEGFR2 (Kim et al. 2002; Ling et al. 2007). Downstream signalling of VEGFR2 is blocked including extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK), p125 focal adhesion kinase ($p125^{FAK}$), and protein B kinase (Akt), resulting in inhibited cell motility, proliferation, and *in vitro* tube formation. Subsequent studies by Ling and colleagues also showed that endostatin induces activation of caspase-3 and decreased Bcl-2 levels, mediating anti-apoptotic effects in HUVECs (Ling et al. 2009).

Endostatin mediates inhibitory effects on angiogenesis via competitively interacting with cell surface expressed proteins such integrin $\alpha_v\beta_3$ and $\alpha_5\beta_1$, and preventing the binding of fibronectin/RGD (Rehn et al. 2001; Sudhakar et al. 2003). Binding of endostatin has been found to inhibit the phosphorylation of focal adhesion kinase (FAK), attenuating downstream signalling pathway ERK, responsible for the ECM-mediated attachment and migratory capacity of ECs (Sudhakar et al. 2003). In addition to integrins, studies by Shi and colleagues identified that endostatin mediates anti-angiogenic and anti-tumour activity through the receptor nucleolin, an 80-kDa protein located on the cell membrane of ECs (Shi et al. 2007). In the presence of endostatin, bFGF- or VEGF-induced phosphorylation of nucleolin is prevented, arresting proliferation of HUVECs and microvascular ECs.

In view of the anti-angiogenic properties of endostatin (Shi et al. 2007; Sim, MacDonald & Gubish 2000), van der Schaft and colleagues tested its effect on VM formation (van der Schaft et al. 2004). Treatment had no effect on VM formation by human melanoma cells *in vitro,* despite its inhibitory action on HUVEC tube formation in an angiogenesis assay (van der Schaft et al. 2004). Similarly, a novel recombinant human endostatin,

endostar, demonstrated minimal effects as a therapeutic strategy against VM formation in glioblastoma (Liu et al. 2011b). Conversely, recent *in vitro* studies showed that endostatin inhibited VM formation by human oesophageal cancer cells as well as proliferation, migration and invasion when used in combination with radiation therapy (Chen et al. 2016).

4.1.2.3 ***Thrombospondin-1***

Thrombospondin-1 (TSP-1) was one of the first endogenous inhibitors of angiogenesis and EC migration identified (Good et al. 1990; Iruela-Arispe, Bornstein & Sage 1991). It is a large homotrimer (150-180-kDa per subunit) glycoprotein, is among the most abundant proteins contained within the α-granules of platelets and is one of the most potent endogenous inhibitors of angiogenesis (Burkhart et al. 2012; Lawler, Slayter & Coligan 1978).

As shown in Figure 4-2, the structure of TSP-1 consists of a heparin binding domain at the N-terminal end, a procollagen homology region and properdin-like types I, II and III regions toward the carboxyl-terminal end (Lawler & Hynes 1986; Tolsma et al. 1993). In addition, TSP-1 also contains an RGD sequence in the type III region and as such TSP-1 can bind to β_3 integrin as well as binding to GPIIb/IIIa (Bonnefoy et al. 2001; Karczewski et al. 1989; Lawler, Weinstein & Hynes 1988). Additional surface receptors for TSP-1 include GPIV (CD36), an 88-kD transmembrane glycoprotein, and integrin-associated protein (IAP; CD47), a 52-kDa transmembrane protein (Asch et al. 1987; Gao et al. 1996). Notably, both CD36 and CD47 are widely expressed in a variety of mammalian cells including platelets and megakaryocytes, ECs and monocytes/macrophages (Berger et al. 1993; Dawson et al. 1997; Febbraio, Hajjar & Silverstein 2001; Gao et al. 1996; Holmes 2012; Lindberg et al. 1993).

Dawson and colleagues were the first to demonstrate that TSP-1 mediates its anti-angiogenic effects through the CD36 receptor (Dawson et al. 1997). However, the down-stream signalling pathways initiated upon TSP-1 binding are not fully understood. TSP-1 is known to mediate anti-angiogenic effects through activating Src-family tyrosine kinase p59fyn, caspase-3 like proteases and p38 mitogen-activated protein kinases (p38MAPK) which culminate to induce apoptosis in ECs (Jimenez et al. 2000). More recently, Chu and colleagues proposed a mechanistic pathway in which TSP-1 signals through CD36 receptor to inhibit VEGF-mediated EC tube formation and migration (Chu, Ramakrishnan & Silverstein 2013). A schematic of this process is depicted in Figure 4-3, illustrating that in the absence of TSP-1, VEGF binds to VEGFR2, inducing phosphorylation of the receptor and thus promoting EC tube formation. When TSP-1 is present and binds to CD36, this results in recruitment of Src homology 2 domain–containing protein tyrosine phosphatase (SHP)-1 which causes dephosphorylation of VEGFR2 and inhibition of EC tube formation. This particular work also confirmed previous studies by Jimenez and colleagues, wherein TSP-1 binding to CD36 initiated activation of Fyn, p38MAPK and caspases resulting in EC apoptosis (Jimenez et al. 2000).

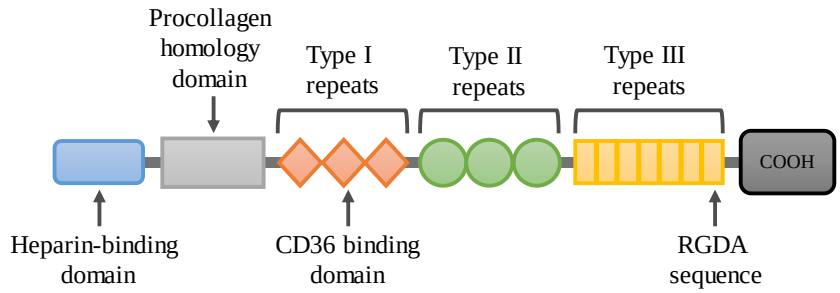


Figure 4-2. Thrombospondin (TSP)-1, subunit structure

Human thrombospondin (TSP-1) consists of a heparin-binding domain, procollagen homology domain, and properdin-like type repeats; 3 type I repeats, 3 type II repeats, and seven type III repeats. Indicated in the last Type III repeat is the RGDA sequence which shares homology with RGD ligands and allows binding of $\alpha_v\beta_3$ integrin. Type I repeats also harbor the CD36 binding domain. Adapted from Lawler and Hynes (1986) and Tolsma et al. (1993).

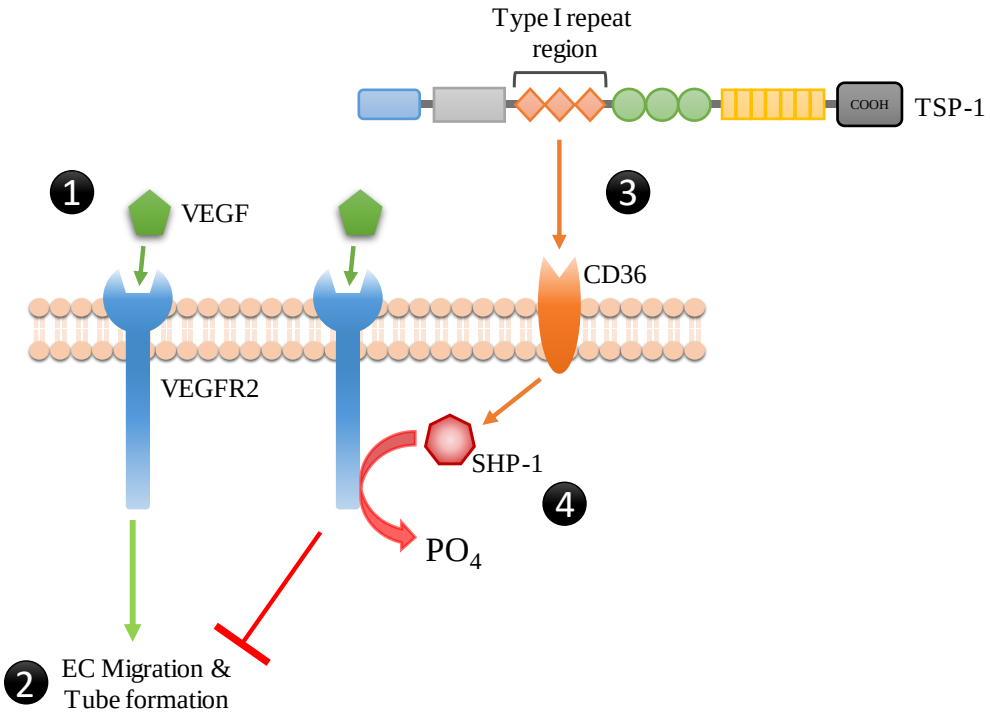


Figure 4-3. The anti-angiogenic effects of TSP-1 through CD36 signalling

In **1**; VEGF signals through VEGF receptor 2 (VEGFR2). In **2**; in the absence of TSP-1, VEGF induces endothelial cell (EC) migration and tube formation. In **3**; TSP-1 (TSP-1) binds to CD36 receptor via the type I repeat region of the molecule, leading to the recruitment of SHP-1. In **4**. SHP-1 dephosphorylates VEGFR2, inhibiting its proangiogenic properties. Adapted from Chu, Ramakrishnan and Silverstein (2013).

It has also been documented that TSP-1 can bind and signal through CD47 to meditate anti-angiogenic effects (Isenberg et al. 2006). Binding of TSP-1 to CD47 inhibits angiogenesis by brain ECs or endothelial progenitor cells *in vitro* (Qin et al. 2014; Xing et al. 2009), and these effects are attenuated when CD47 is silenced via siRNA (Qin et al. 2014). TSP-1 binding to CD47 impairs pro-angiogenic signalling by attenuating nitric oxide (NO)/cGMP signalling (Isenberg et al. 2009; Roberts et al. 2007). Similar to TSP-1/CD36 signalling, recent studies also showed that binding of TSP-1 to CD47 inhibits VEGF-induced VEGFR2 phosphorylation in bovine aortic ECs as well as microvascular ECs (Kaur et al. 2010).

In addition to normal angiogenesis, TSP-1 also attenuates tumour angiogenesis, an effect demonstrated using *in vivo* mouse models of melanoma, breast cancer, lung carcinoma, testicular carcinoma and fibrosarcoma (Filleur et al. 2001; Hale et al. 2012; Lawler et al. 2001; Volpert, Lawler & Bouck 1998; Yee et al. 2009). For example, TSP-1 null mice inoculated with B16/F10 melanomas exhibited a 2-fold greater rate of cancer growth with increased tumour vascularisation, elevated proliferation and reduced apoptosis compared to wild-type (WT) mice (Lawler et al. 2001). Similarly, in the polyomavirus middle T (Pyt) mouse breast cancer model, tumours were more advanced and grew more rapidly in TSP-1 null mice compared to wildtype mice (Yee et al. 2009). The TSP-1-null mice had fewer vessels within the tumours at 60 days post-inoculation and they were significantly larger compared to wildtype mice. Despite the effective inhibitory properties of TSP-1 in both physiological as well as pathological angiogenesis, it is not known if TSP-1 also regulates VM by cancer cells.

In Chapter 3, it was shown that platelets inhibited VM formation in the C32 melanoma cell line as well as the breast cancer cell lines MDA-MB-231 and HS-578T. Strikingly, platelets were unable to inhibit VM formation by the CHL-1 melanoma cell line. Chapter 4 investigates a potential mechanism through which platelets may mediate VM formation *in vitro*. It is hypothesised that the anti-VM effect of platelets is also mediated by one of the anti-angiogenic factors produced by platelets, and that this factor binds to a surface expressed protein on the cancer cells. To test this, the phenotypic profile of the various cancer cell lines was investigated and compared using publicly available gene expression profiles of surface expressed proteins as well as flow cytometric analysis. The effect of these proteins was then interrogated via small interfering RNA on platelet mediated VM inhibition using the *in vitro* tube formation assay.

4.2 Materials and methods

4.2.1 Collection of platelet-released contents

Human platelets were isolated and purified as previously described in Chapter 2 (section 2.3.3). Washed platelets were adjusted to ~100x10^6/mL and activated with 0.5 International Units (IU) human α-thrombin (section 2.3.4). To confirm platelet activation, CD62P expression was measured via flow cytometry (section 2.4.1.1). Samples were centrifuged (2,800xg for 10min) and supernatant (containing platelet-released contents) collected, aliquoted and stored at -20°C for later use in co-culture tube formation studies.

4.2.2 Tumour-conditioned platelet medium

For the preparation of tumour-conditioned platelet medium (CM), C32 (5x10^5), and CHL-1 (1.75x10^5) melanoma cells were plated in 24-well plates and allowed to adhere overnight. The following day, the medium was removed and replaced with 1mL of fresh culture medium as well as 1mL of purified platelets in Tyrode's wash buffer at either 40-fold or 200-fold the cancer cell concentration. Control wells received 1mL Tyrode's wash buffer alone. The cells ± platelets were incubated for 15 min at 37°C in 5% CO_2 after which time the CM was collected and centrifuged at 2,800xg for 10 min to pellet any platelets or cancer cells. Aliquots of the CM were then stored at -20^{o}C until used.

4.2.3 Scratch wound assays

Cancer cells were grown to a confluent monolayer on flat-bottom 96-well plates. Upon reaching confluence, a 96-pin wound making tool (WoundMaker™) was used to create 700-800µm wide scratch wounds in the cancer cell monolayers. After making the scratch wound, wells were washed twice with complete medium to remove all non-adherent cells.

For these experiments, purified platelets (section 2.3.3), or CM (see Section 4.2.2) were used as described below. Control wells received buffer (Tyrode's wash buffer in 80µL complete media), and test samples received concentrations of 1:40 platelets, 1:40 CM or 1:200 CM (20µl CM in 80µL complete media). Plates were incubated at 37°C in 5% CO_2, for 24 hours. Real-time cancer cell migration was assessed by phase contrast photomicrographs collected every 15min using the Incucyte Zoom system (Essen, Michigan, USA) with 10x objective. Wound closure was measured over time using ImageJ software (National Institute of Health, USA).

4.2.4 *In silico* gene expression

The publically available Cancer Cell Line Encyclopedia (CCLE) microarray dataset was utilised to investigate genes of interest in various cancer cell lines (Barretina et al. 2012). The CCLE dataset GSE36133 was accessed via the NCBI Gene Expression Omnibus (GEO) and interrogated using the GEO2R webtool (https://www.ncbi.nlm.nih.gov/geo/geo2r/) to generate relative expression values for breast cancer cell lines and melanoma cell lines. Analysis was performed on a panel of 56 breast cancer cell lines including MDA-MB-231 and HS-578T, and 58 melanoma cell lines including C32 and CHL-1.

4.2.5 **Flow cytometric analysis**

Expression of surface antigens on cancer cell lines was assessed using flow cytometry as described in detail previously (section 2.4.1.2). Briefly, $1x10^6$ cells were stained with anti-PSGL-1 antibody diluted 1:100, anti-integrin $\alpha_v\beta_3$ antibody diluted 1:100, anti-CD36 antibody diluted at 1:2000 (Table 2-1) or relevant isotype controls. Samples were then stained with secondary antibody DyLight650 (Table 2-1) and with dead cell exclusion dye 7-AAD (Table 2-5). Data were collected using a BD Accuri C6 flow cytometer (Ann Arbor, MI, USA) and analysis performed on FCS Express 6 software (De Novo Software, Glendale, CA).

4.2.6 **Protein knockdown with small interfering RNA**

Cell transfections with small interfering RNA (siRNA) were performed in T25 flasks and 12-well plates as per manufacturer's instructions (detailed method in section 2.4.2). Briefly, cancer cells were transfected with 1) untreated control, 2) scrambled-siRNA, or target specific 3) siRNA-A, 4) siRNA-B, and 5) siRNA-C. Following transfection, plates and flasks were incubated for 48 hours at 37°C in 5% CO_2. Knockdown at the protein level was validated by flow cytometric analysis (section 2.4.1.2).

4.2.6.1 ***VM formation assay***

Thawed supernatants were used only once to avoid freeze-thaw variations and were used in the VM tube formation assay in ratios of (cancer cell: supernatant) 1:0 (control or buffer only), 1:2, 1:5, 1:10, 1:20, and 1:40. The supernatant represents the released contents collected from platelets, for example, the 1:40 ratio was prepared as one cancer cell plus the supernatant collected from 40 platelets. In each sample, the final volume was always the same. Tube formation assays were performed as previously described in Chapter 2

(section 2.3.5). For time-lapse video microscopy, images were captured using the CellVoyager™ CV1000 Confocal scanner system and software (Yokogawa, Tokyo, Japan) using 10x objective.

The effect of thrombospondin (TSP-1) on C32 melanoma cells was also investigated using the tube formation assay. Transfected cells were treated with 10nM TSP-1 (Table 2-5) or PBS in 12-well plates for 4 hours at 37°C in 5% CO_2. Incubation time and concentration of TSP-1 were as previously published (Chu, Ramakrishnan & Silverstein 2013). Cells were then washed with fresh medium, harvested with 0.1M EDTA in PBS and centrifuged at 400xg for 5 min. Viable cells were identified using trypan blue staining and counted prior to $2x10^5$ cells being seeded onto Geltrex coated angiogenesis well slides (previously described, section 2.3.5) then incubated at 37°C in 5% CO_2. Images were collected after 5 hours using Olympus CKX-41 Inverted Microscope (Tokyo, Japan) with 4x objective, fitted with a DP70 digital camera and operated through the Olympus Image Analysis Cell B software program (Olympus, Tokyo, Japan).

4.2.7 **Statistical analysis**

Unless otherwise indicated, results were expressed as mean ± standard error of the mean (SEM) from at least 3 experiments. Statistical analysis was performed using Graphpad software. As indicated in the figure legends, data was analysed with Student's t-test, or one-way ANOVA to determine statistical significance with p value <0.05.

4.3 Results

4.3.1 The effect of platelets on stages of VM network formation

To further investigate the effect of platelets on VM formation by cancer cells, time lapse video imaging using the C32 and CHL-1 melanoma cell lines was compared. These two melanoma cell lines were specifically chosen based on the results in Chapter 3 wherein platelets differentially affected VM formation with an inhibition observed on the C32 melanoma cell line and no effect on the CHL-1 melanoma cell line.

As shown in the schematic of Figure 4-4 (**A**), the process of VM formation by cancer cells *in vitro* occurs via a cascade of events. These include: Stage 1, wherein cells seeded onto Geltrex matrix immediately settle onto the underlying ECM as a single cell suspension and begin to change shape and make contact with neighbouring cells; Stage 2 (~1-2 hours post seeding) wherein cells elongate in length and increase their level of interconnectedness and Stage 3 (~4 hours) wherein the cancer cells form multi-cellular inter-connected vascular-like structures. Notably, these processes were equally observed for both melanoma cell lines C32 and CHL-1 during VM formation (Figure 4-4, **B**).

When platelets were seeded at a ratio of 1:40 (cell: platelets), both C32 and CHL-1 cancer cells were found to undergo Stage 1 of VM formation (i.e. single cell gathering and interaction), but at Stage 2, C32 melanoma cells were unable to migrate and extend in length and were thus inhibited from forming VM structures (Figure 4-5; **A**). Conversely, CHL-1 melanoma cells retained their capacity to form VM structures in the presence of platelets (Figure 4-5; **B**).

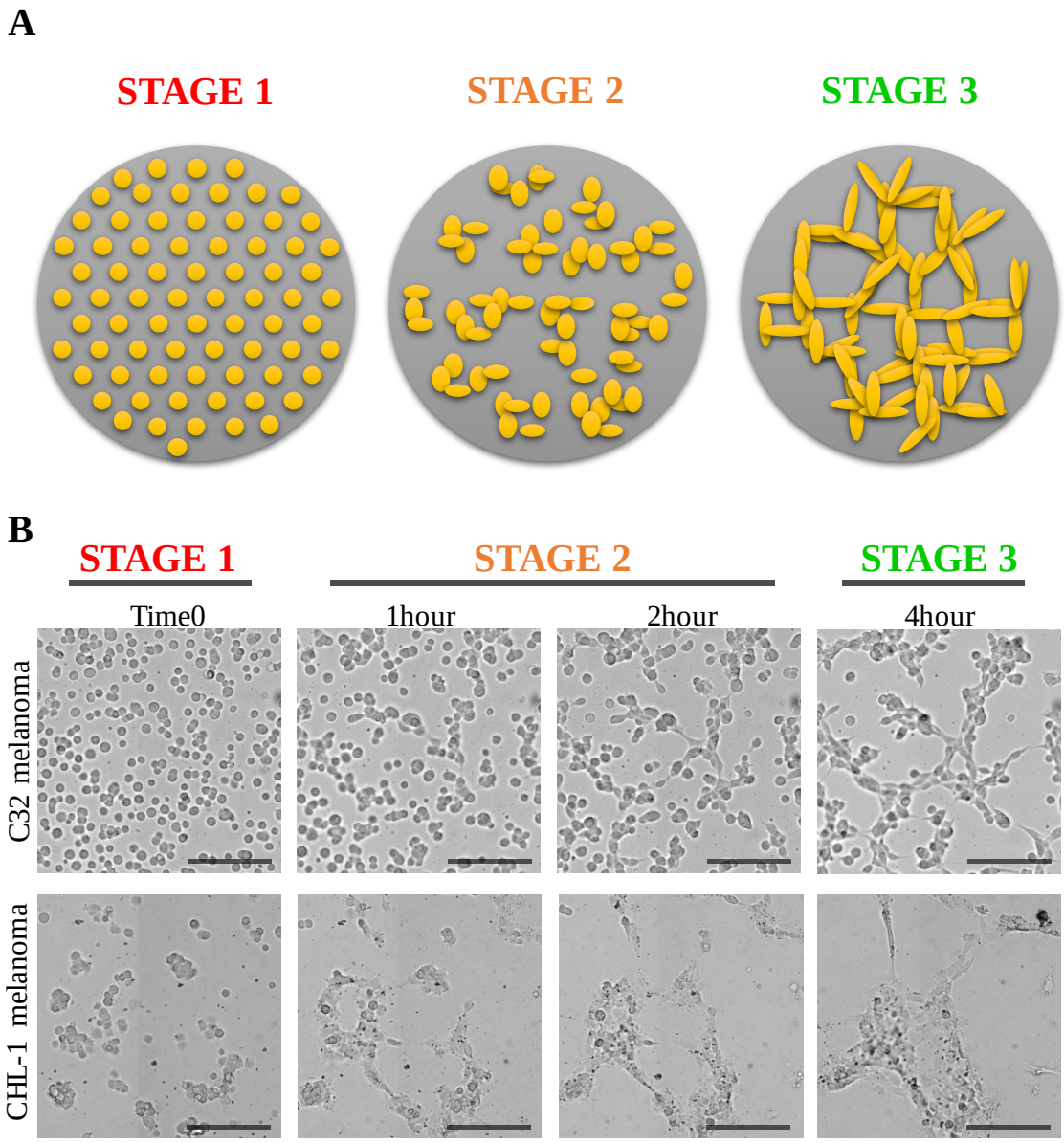


Figure 4-4. Stages of VM network formation

The process of VM was captured by video microscopy using the C32 and CHL-1 melanoma cancer cell lines over a period of 4-5 hours. The process can be separated into various stages (**A**-**B**); (Stage 1) cancer cells begin as single cells which change shape and begin to make contact with neighbouring cells, (Stage 2) cells elongate in length and begin to connect at 1-2 hours of incubation, and (Stage 3) inter-connected vascular-like structures are formed by four hours of seeding. Scale bar is 200µm, original magnification 100x.

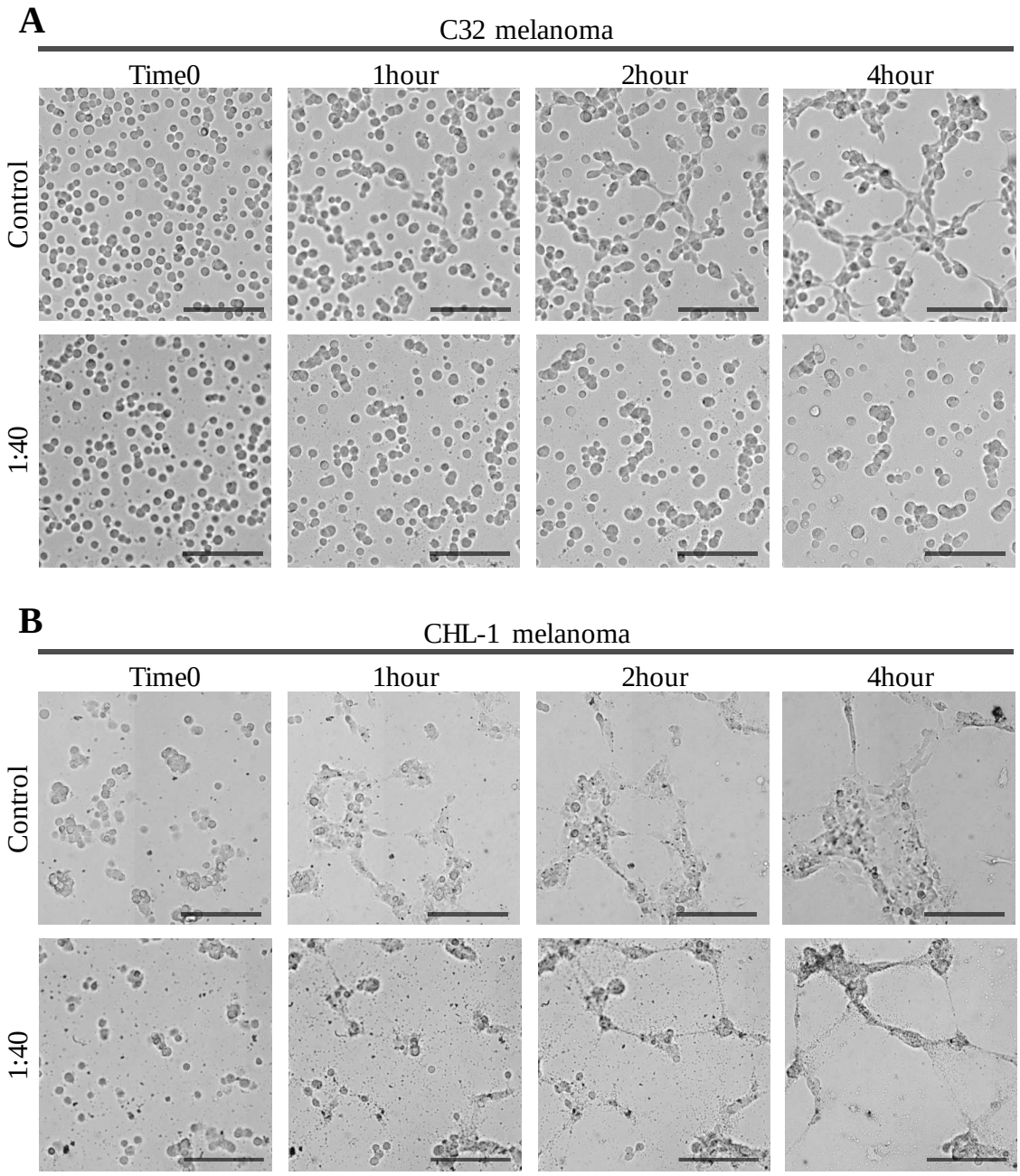


Figure 4-5. Platelet selectively inhibit cancer cell VM in melanoma cell lines

Time lapse imaging of VM formation by (**A**) the C32 melanoma cell line and (**B**) the CHL-1 melanoma cell line upon treatment with buffer control or ratio of cancer cell: platelets of 1:40. Representative time frames of a single experiment. Scale bar is 200µm, original magnification 100x.

4.3.2 **Effect of platelet released contents on VM network formation**

To decipher the mechanism by which platelets inhibit VM formation by the C32 melanoma cell line, the potential contribution of a soluble factor released from platelets was investigated. To do this, purified platelets were activated by human α-thrombin which caused the platelet to release their granule contents. As shown by flow cytometric analysis in Figure 4-6 (**A**), platelets were first gated using a forward scatter and side scatter profile, and then investigated for the expression of platelet activation marker CD62P (Figure 4-6; **B**). As expected, with increasing concentrations of α-thrombin, a dose dependent activation of platelets occurred as indicated by an increase in the surface expression of CD62P when compared to the baseline (untreated platelets) (Figure 4-6; **C**). Notably, maximum expression of CD62P (~85% of the platelet population) was obtained using 0.5 IU α-thrombin.

To assess the effect of platelet-released contents (hereafter called 'platelet releasate') on VM formation by cancer cells, the Geltrex angiogenesis assay was used. The C32 melanoma and HS-578T breast cancer cell lines were selected according to the results in Chapter 3 wherein VM formation was inhibited in both cell lines when platelets were administered. As shown in Figure 4-7 (**A, C**), C32 melanoma cells co-cultured with platelet releasate resulted in a significant inhibition of VM formation ($P<0.01$). Similarly, VM formation by HS-578T breast cancers cells was also inhibited when exposed to platelet releasate ($P<0.05$) (Figure 4-7; **B, D**).

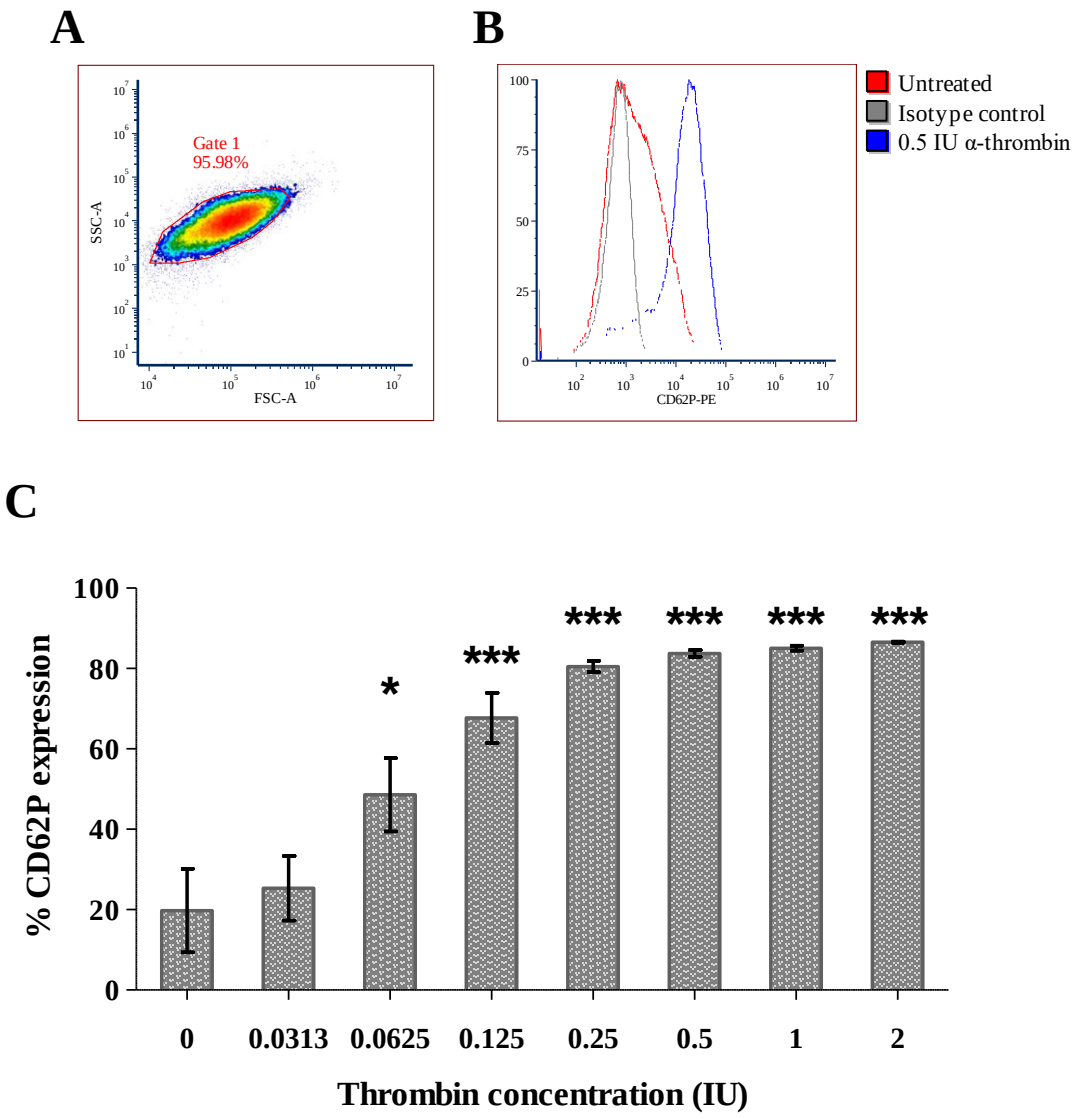


Figure 4-6. Thrombin activation of washed platelets

Platelets purified from healthy human donors were assessed for activation following incubation with human α-thrombin at the indicated concentrations (international unit; IU) for 5 min at 37°C using flow cytometry. (**A**) Representative flow cytometry scatter plot showing the gated platelet population by forward scatter and side scatter. (**B**) The population in Gate 1 was stained for expression of CD62P. Curves represent (red) untreated platelets, (grey) isotype control, and (blue) platelets treated with 0.5 IU α-thrombin. (**C**) Percent expression of platelets stained positive for CD62P. Data are expressed as mean ± SEM. Results are pooled from 3 separate experiments (different donors). **P*<0.05, ****P*<0.001 vs no treatment control, one-way ANOVA with Bonferroni's post-test.

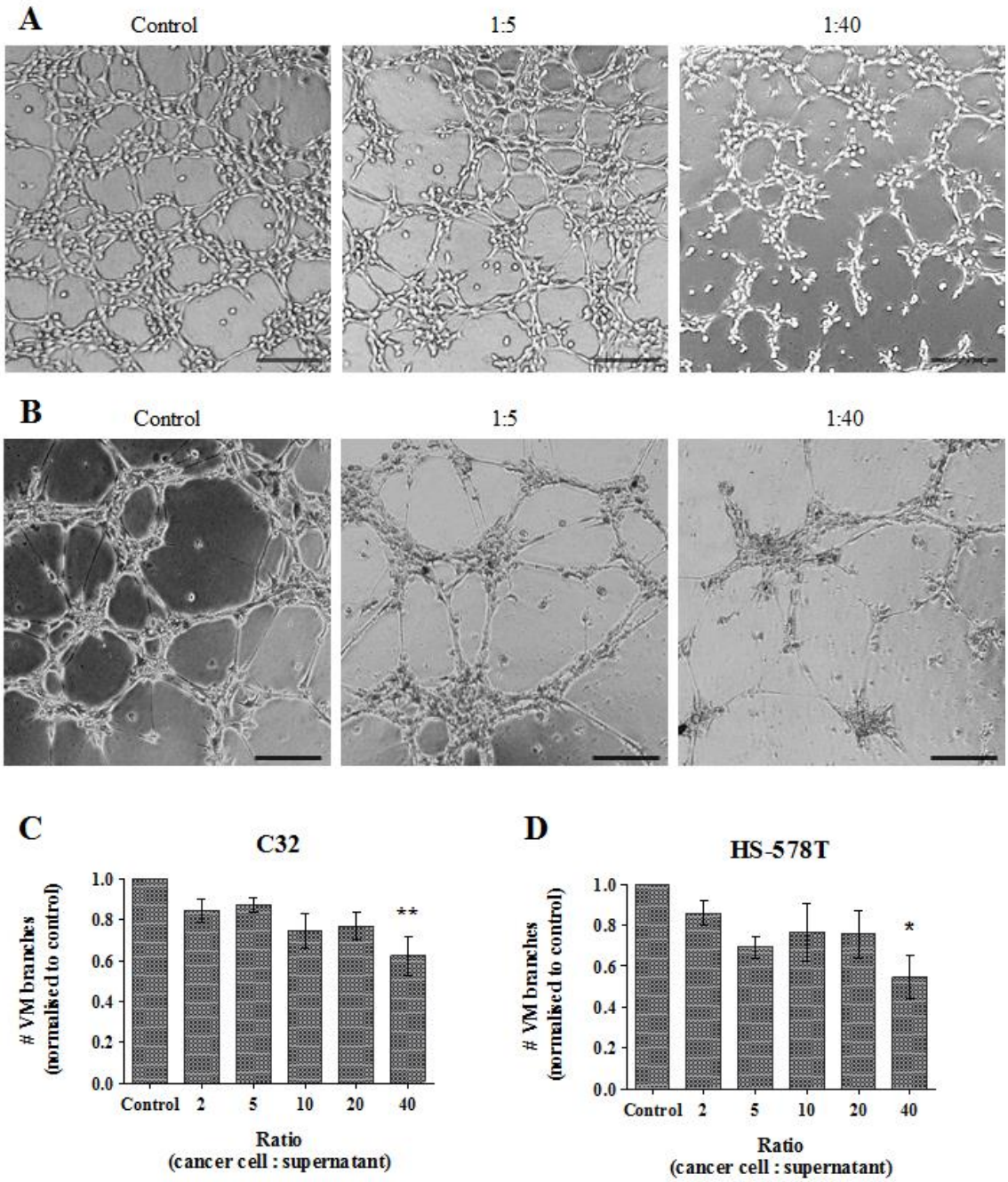


Figure 4-7. Release content from thrombin-activated platelets can attenuate the ability of cancer cell to form VM *in vitro*

(**A**) Representative images of C32 melanoma cells undergoing VM without and with co-culture of α-thrombin-activated platelet releasate at the indicated ratio (cells: supernatant). The supernatant is the released contents from the respective number of platelets. Similarly (**B**) HS-578T breast cancer cells treated with buffer control or releasate from platelets. (**C**) VM formation by C32 melanoma and (**D**) HS-578T breast cancer cells was quantified following treatment with increasing platelet supernatant. Data are expressed as mean ± SEM. Results are pooled from 3 separate experiments (different donors). **P*<0.05, ***P*<0.01, one-way ANOVA. Scale bar is 200µm, original magnification 40x.

4.3.3 **Migratory capacity of cancer cells**

Having confirmed through video time-lapse microscopy that platelets inhibit VM formation by blocking the elongation of VM structures, the effect of platelets and their components on the migratory capacity of cancer cells was then investigated. CHL-1 and C32 melanoma cell lines were directly compared based on previous results that showed VM formation by C32 melanoma cells to be inhibited by platelets, but CHL-1 melanoma cell VM formation was not. Using the scratch wound assay, in the presence of platelets, scratch wound healing by C32 melanoma cells was inhibited at 12 hours ($P<0.05$) and at 24 hours ($P<0.01$) when compared to buffer control (Figure 4-8; **A**, **C**). Conversely, no statistically significant difference in scratch wound closure was observed with exposure of CHL-1 melanoma cells to platelets, even at 24 hours ($P<0.082$) (Figure 4-8; **B**, **D**). These results suggest that platelets inhibit the migration of C32 but not CHL-1 melanoma cells, and support the earlier observation of interrupted VM formation by some, but not all, melanoma cells.

The effect of platelet components was also investigated using the scratch wound assay. In previous experiments (Figure 4-7), platelet releasate was generated by activating platelets with α-thrombin. However, to establish a more clinically relevant scenario of platelet-cancer cell interactions, the releasate from platelets was induced by exposing platelets to cancer cells. Herein this platelet releasate is referred to as tumour-conditioned platelet medium (CM). As shown in Figure 4-8 (**E-F**), CM had no effect on scratch wound healing by either C32 or CHL-1 melanoma cells.

4.3.4 **Gene expression of markers of cancer cell-platelet interactions**

Having shown that platelets exert a differential effect on VM by different cancer cell lines with a notable lack of effect on the CHL-1 melanoma cell line, an *in silico* gene expression analysis of the CCLE microarray dataset was then conducted to elucidate whether the effect was associated with differential expression of markers involved in cancer cell-platelet interactions.

First, the expression of genes previously documented to promote platelet-cancer cell interactions, including PSGL-1 (*SELPLG*) and integrin $\alpha_v\beta_3$ subunits (*ITGAV* and *ITGB3*) were examined. As shown in Figure 4-9 (**A**), the C32 melanoma cell line exhibited the highest gene expression of *ITGB3*. In comparison, *ITGB3* displayed modest expression in the HS-578T breast cancer cell line, and low expression in the MDA-MB-231 breast cancer cell line and CHL-1 melanoma cell line. Also shown in Figure 4-9 (**A**), *ITGAV* was expressed highly across all cell lines and *SELPLG* was expressed in low levels across all cell lines. These findings suggest that C32 melanoma cells may form stronger interactions with platelets via the integrin *ITGB3* when compared to MDA-MB-231, HS-578T, and CHL-1 cancer cell lines.

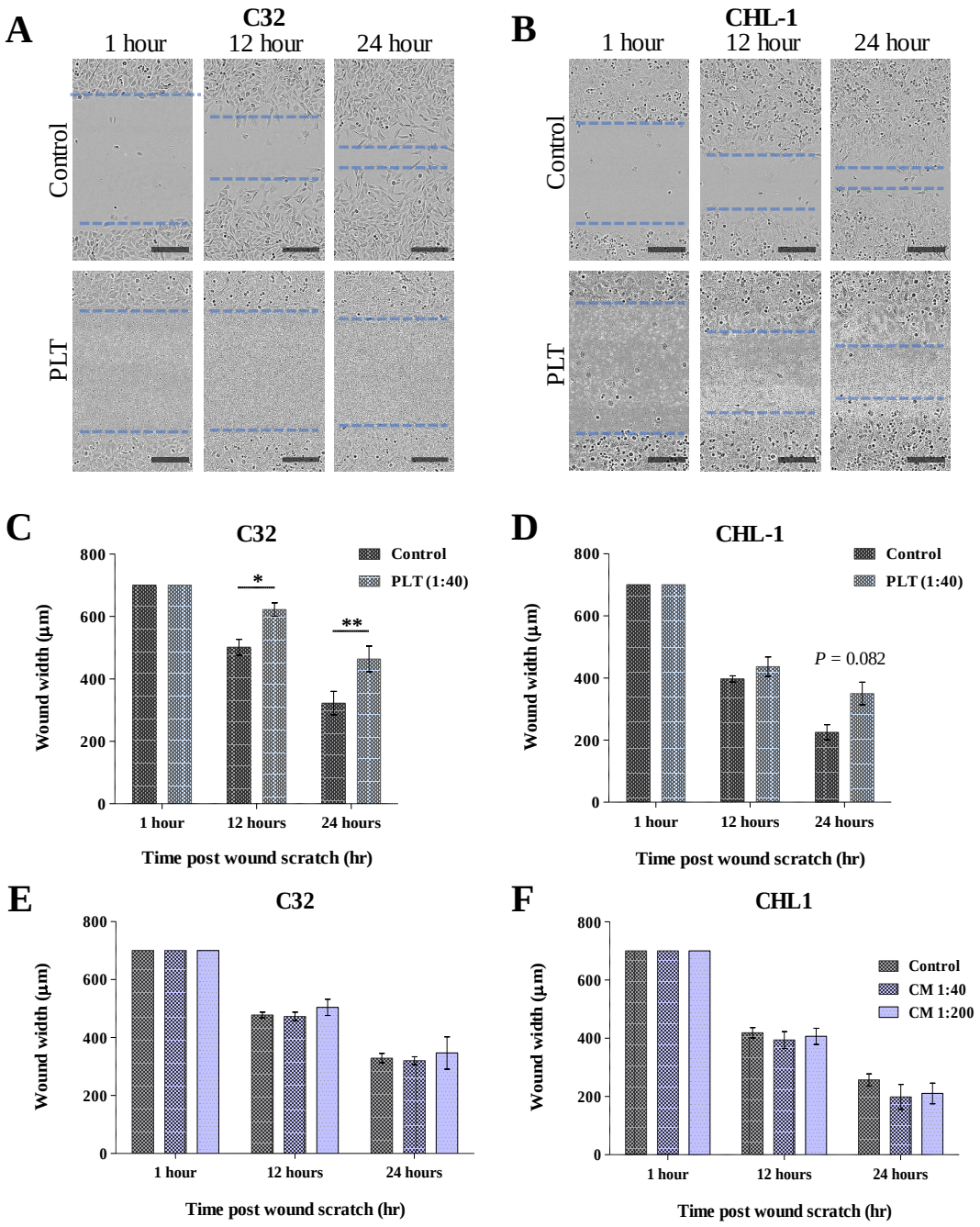


Figure 4-8. Platelets and tumour-conditioned platelet medium (CM) differentially affect scratch wound closure by cancer cell lines

(**A**) Representative images showing scratch wound assay by C32 melanoma cells and (**B**) CHL-1 melanoma cells treated with buffer control or platelets (PLT) at a cancer cell: platelet ratio of 1:40 at the indicated time points. Dotted lines indicate wound margin. (**C-D**) Quantified scratch wound healing by melanoma cells treated with buffer control or platelets. (**E-F**) Quantified wound healing by melanoma cells treated with control or CM. Data are expressed as mean ± SEM. Results are pooled from 3 separate experiments (different donors). **P*<0.05, ***P*<0.01, two-way ANOVA with repeated measures. Scale bar is 200µm, original magnification 100x.

The expression of platelet agonist markers, prothrombin (*F2*; coagulation factor II) and tissue factor (*F3*; coagulation factor III) by cancer cells was then investigated. As shown in Figure 4-9; (**B**), the expression of *F2* was similar across cancer cell lines. In contrast to *F2*, *F3* was elevated in all cancer cell lines and more highly expressed in MDA-MB-231 and HS-578T. Collectively, these findings suggest that although there are similarities in expression of platelet receptor subunits and coagulation proteins between cancer cell lines, differences in expression of proteins which support platelet-cancer cell interactions do occur and may explain the differing effects observed with respect to the role of platelets in VM.

4.3.5 **Identification of a mechanistic pathway**

Since platelet releasate (α-thrombin activation) inhibited VM formation (Figure 4-7), and cancer cells show similar expression of platelet agonists (Figure 4-9), *in silico* gene expression analysis was undertaken to determine whether cancer cells differentially express receptors to anti-angiogenic factors released from platelets. As platelets are a source of angiogenesis inhibitors including angiostatin, endostatin and TSP-1, differential expression of the receptors between cancer cell lines may explain the selective platelet anti-VM effect. The expression of angiostatin receptor annexin II (*ANAX2*) (Figure 4-10; **A**), and endostatin receptor nucleolin (*NCL*) (Figure 4-10; **B**) genes was assessed and found to be high in all four cancer cell lines.

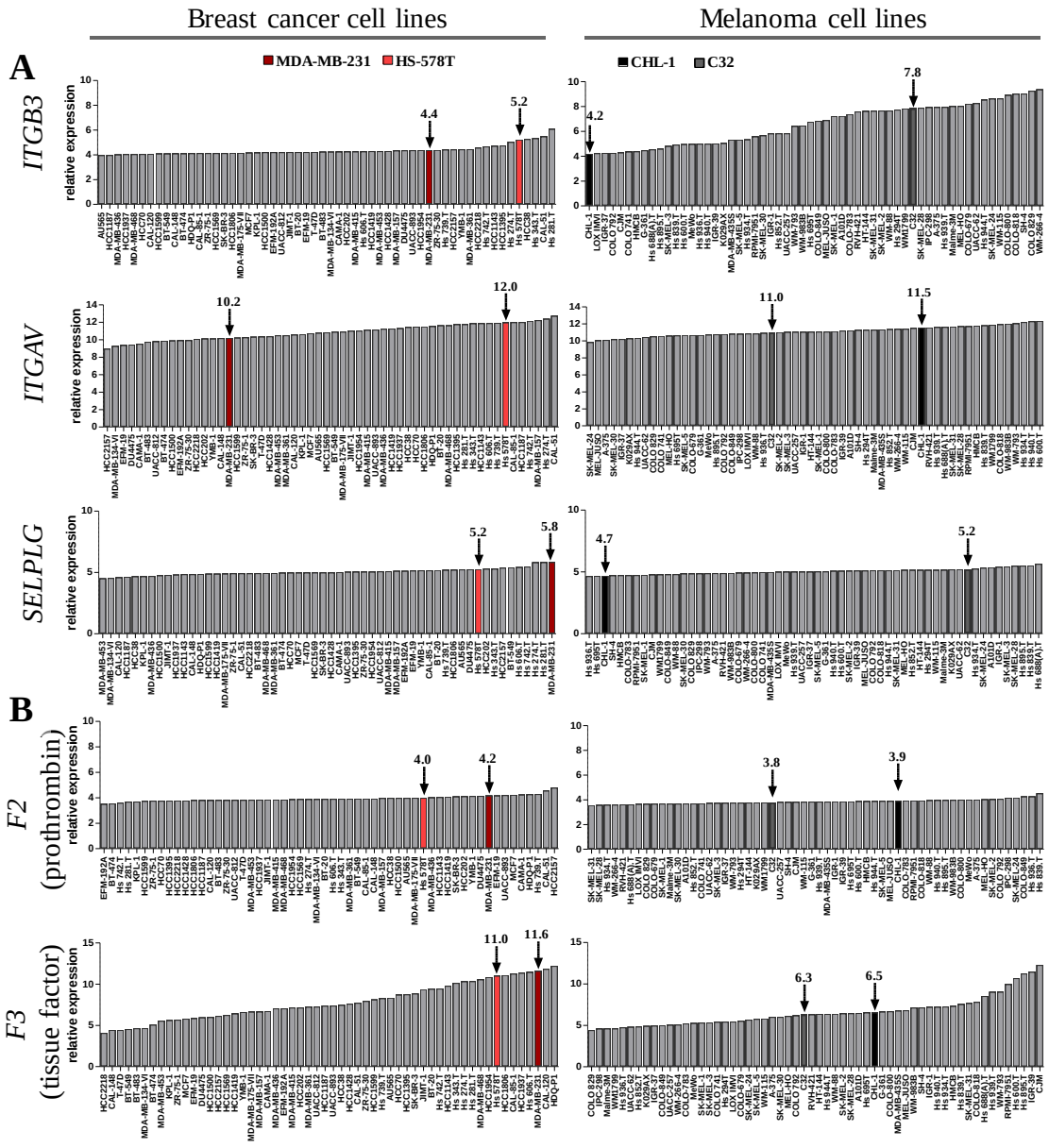


Figure 4-9. Melanoma and breast cancer cells lines heterogeneously express genes involved in cancer cell-platelet interactions

The Cancer Cell Line Encyclopedia (CCLE) gene expression microarray dataset (GSE36133) was interrogated using GEO2R to generate relative expression values for breast cancer (n = 56) and melanoma cell lines in the database (n = 58). (**A**) Expression of genes involved in mediating cancer cell adhesion to platelets; integrin β_3 (*ITGB3*), integrin α_v (*ITGAV*), and P-selectin glycoprotein ligand-1 (*SELPLG*). (**B**) Expression of genes involved in mediating platelet activation; *F2* (coagulation factor II; prothrombin), *F3* (coagulation factor III, tissue factor). Red coloured bars indicate breast cancer cell lines (MDA-MB-231 and HS-578T) and black coloured bars indicate melanoma cell lines (C32 and CHL-1). Black arrow indicates relative expression.

Relative expression of *CD47* was similar across C32 melanoma cell line, MDA-MB-231 and HS-578T breast cancer cell lines, whilst CHL-1 showed the lowest expression in comparison to all breast cancer and melanoma cell lines in the database. Similarly, high expression of *CD36* was observed in the C32 melanoma cell line, whilst low expression was observed in CHL-1 melanoma cell line as well as breast cancer lines MDA-MB-231 and HS-578T (Figure 4-10; **C**). This suggests that the C32 melanoma cell line could potentially express significantly higher levels of CD36, a receptor for platelet released anti-angiogenic factor TSP-1 than the CHL-1 melanoma cell line. Given that binding of TSP-1 to its receptor CD36 is shown to inhibit VEGFR2-induced tube formation in MVEC by causing dephosphorylation (Chu, Ramakrishnan & Silverstein 2013), *in silico* gene expression analysis was also used to assess VEGFR2 expression in cancer cells. Interestingly, in C32 melanoma cells, VEGFR2 (*KDR*) was also expressed at high levels, approximately double that expressed by the CHL-1 melanoma cell line (Figure 4-9; **D**). Collectively, the gene expression studies suggest that the C32 melanoma cells expresses high levels of genes which are specifically related to cancer cell platelet-interactions that may promote a response to TSP-1, an abundant anti-angiogenic factor released from platelets.

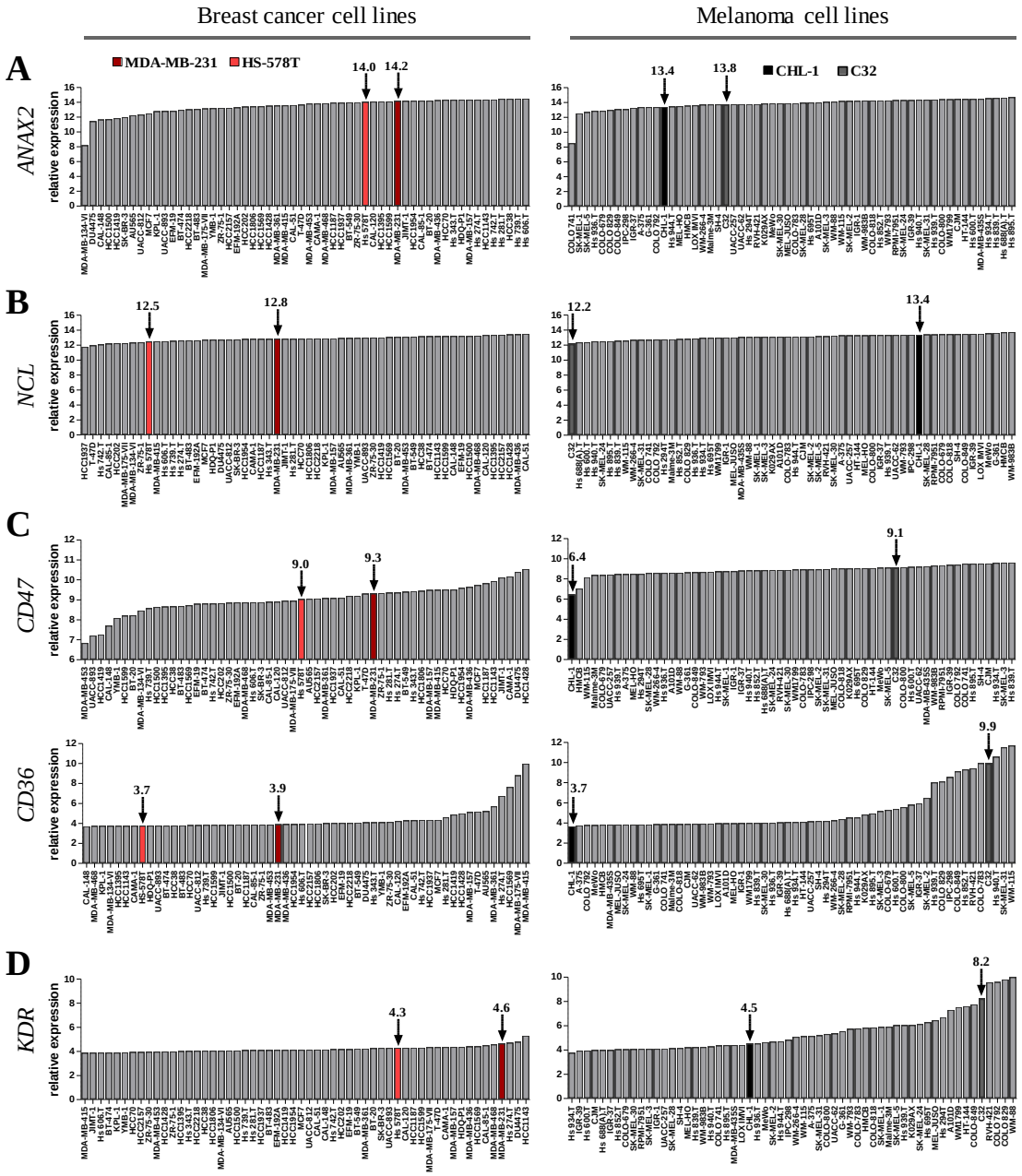


Figure 4-10. The CD36/TSP-1 pathway is highly expressed in the C32 melanoma cell line

The Cancer Cell Line Encyclopedia (CCLE) gene expression microarray dataset (GSE36133) was interrogated using GEO2R to generate relative expression values for breast cancer (n = 56) and melanoma cell lines in the database (n = 58). In (**A**); expression of *ANAX2* (Annexin A2; angiostatin receptor). (**B**) Expression of *NCL* (Nucleolin; endostatin receptor). (**C**) Expression of *CD47* and *CD36* (TSP-1 receptors). (**D**) Expression of *KDR* (VEGFR-2). Red coloured bars indicate breast cancer cell lines (MDA-MB-231 and HS-578T) and black coloured bars indicate melanoma cell lines (C32 and CHL-1). Black arrow indicates relative expression

4.3.6 **Protein expression of cancer cell-platelet interaction markers on cancer cells**

To validate gene expression data obtained from the CCLE database, markers of interest were further investigated for their expression at the cell surface using flow cytometry. It is well documented that cancer cell-platelet interactions can occur through integrin $\alpha_v\beta_3$ and GPIIb/IIIa on the cancer cells and platelets, respectively; as well as PSGL-1 and P-selectin on the cancer cells and platelets, respectively (Felding-Habermann et al. 1996; Kim et al. 1998a). As shown in Figure 4-11, the mean fluorescence intensity (MFI) values indicate that the C32 melanoma cell line expressed significantly higher levels of integrin $\alpha_v\beta_3$ at the cell surface, whilst HS-578T breast cancer cells showed modest levels of expression. Expression by MDA-MB-231 and CHL-1 cancer cell lines was negligible (Figure 4-11; **A**). Notably, the surface expression of PSGL-1 expression was very low across all cancer cell lines (Figure 4-11; **B**).

Of all four cancer cell lines examined, the C32 melanoma cell line demonstrated the highest level of sensitivity to the anti-VM effects of platelets (Chapter 3, Figure 4-5 and Figure 4-7), and gene expression analysis suggested a potential pathway via the TSP-1/CD36 axis. As anticipated, and similar to the gene expression data, CD36 surface protein was most highly expressed by the C32 melanoma cancer cell line, whilst expression on all other cancer cell lines was negligible (Figure 4-11; **C**). These studies further support the potential signalling pathway between TSP-1 and CD36 as a contributor to the anti-VM effect of platelets by C32 melanoma cells.

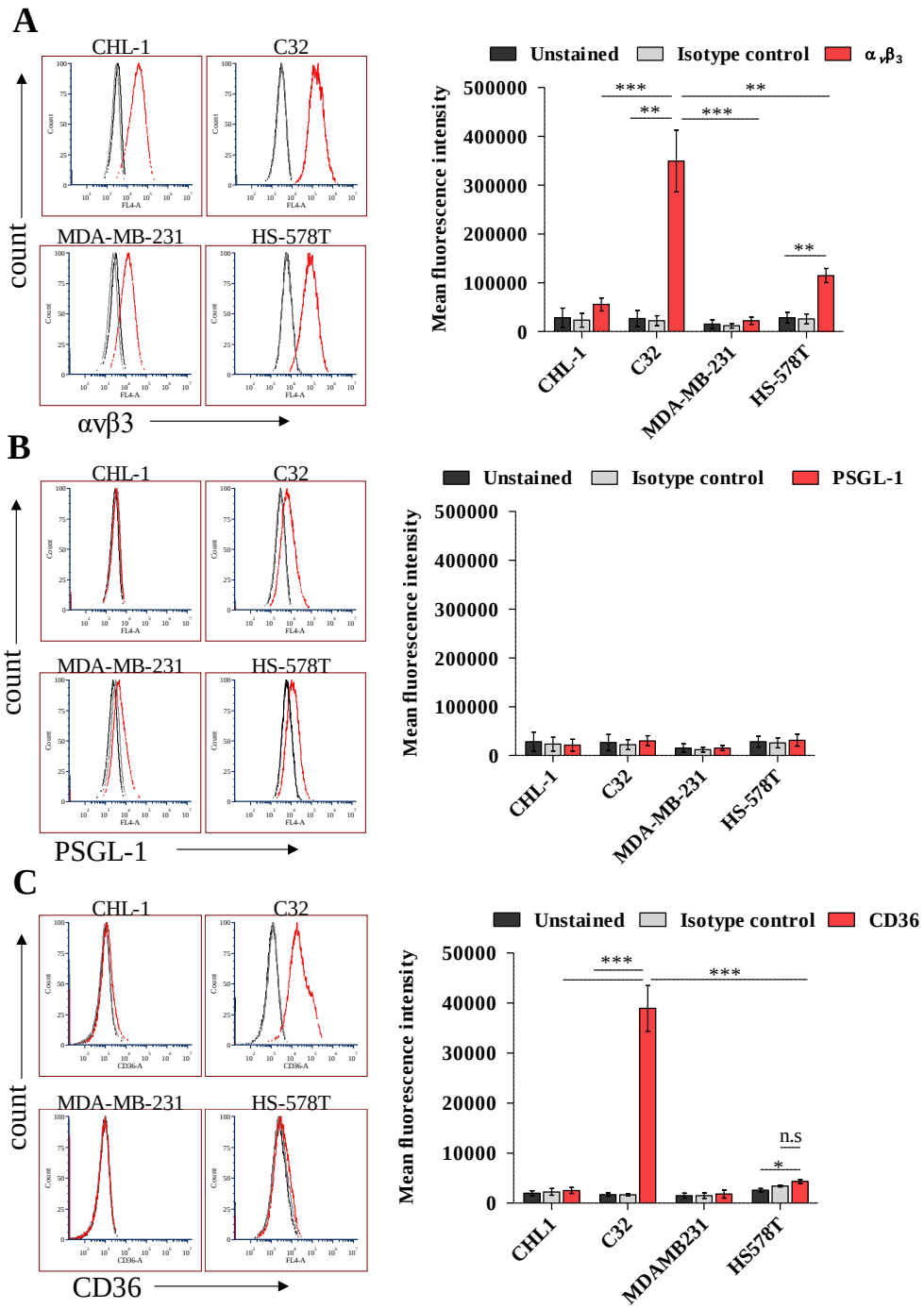


Figure 4-11. Flow cytometric analysis of surface expressed molecules in human cancer cell lines

Flow cytometric analysis revealed the expression of (**A**) integrin $\alpha_v\beta_3$, (**B**) PSGL-1, and (**C**) CD36. The mean fluorescence intensity was assessed in the melanoma cell lines (C32 and CHL-1) as well as the breast cancer cell lines (MDA-MB-231 and HS-578T). Curves represent (black) unstained cells, (grey) isotype control, and (red) integrin $\alpha_v\beta_3$, PSGL-1, or CD36. The data are quantified in the right panels, expressed as mean ± SEM. Results are an average of three separate experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 vs isotype control, one-way ANOVA with Bonferroni's post-test.

4.3.7 **The role of the CD36/TSP-1 pathway in VM formation**

To investigate whether the TSP-1/CD36 pathway is involved in mediating the platelet-anti-VM effects on the C32 melanoma cell line, the tube formation assay was utilised to assess VM formation following manipulation of the pathway. The effect of platelet-derived TSP-1 was investigated on CD36-expressing C32 cells by comparing to cells wherein the CD36 expression was knocked down via gene silencing using siRNA. Flow cytometric analysis was used to evaluate expression levels.

Cells were transfected with scrambled-siRNA (C32-scram) or three different CD36-targeting siRNA constructs (siRNA-A, -B, and -C) in various doses (1nM, 5nM or 10nM) (Figure A2-1; **A**) and protein expression was assessed at various times (24, 48, and 72 hours) by flow cytometry (Figure A2-1; **B**). These optimisation studies determined that the best knockdown was achieved at 48 hours with 1nM siRNA. As illustrated in Figure 4-12, these conditions resulted in a significant reduction in CD36 expression in the C32 melanoma cell line using all three siRNAs, as indicated by MFI (Figure 4-12; **A**-**B**). All siRNAs were equally effective (>70% knockdown), so for future experiments one siRNA (siRNA-B) was used to knockdown CD36 expression in C32 melanoma cells (C32-$CD36^{KD}$).

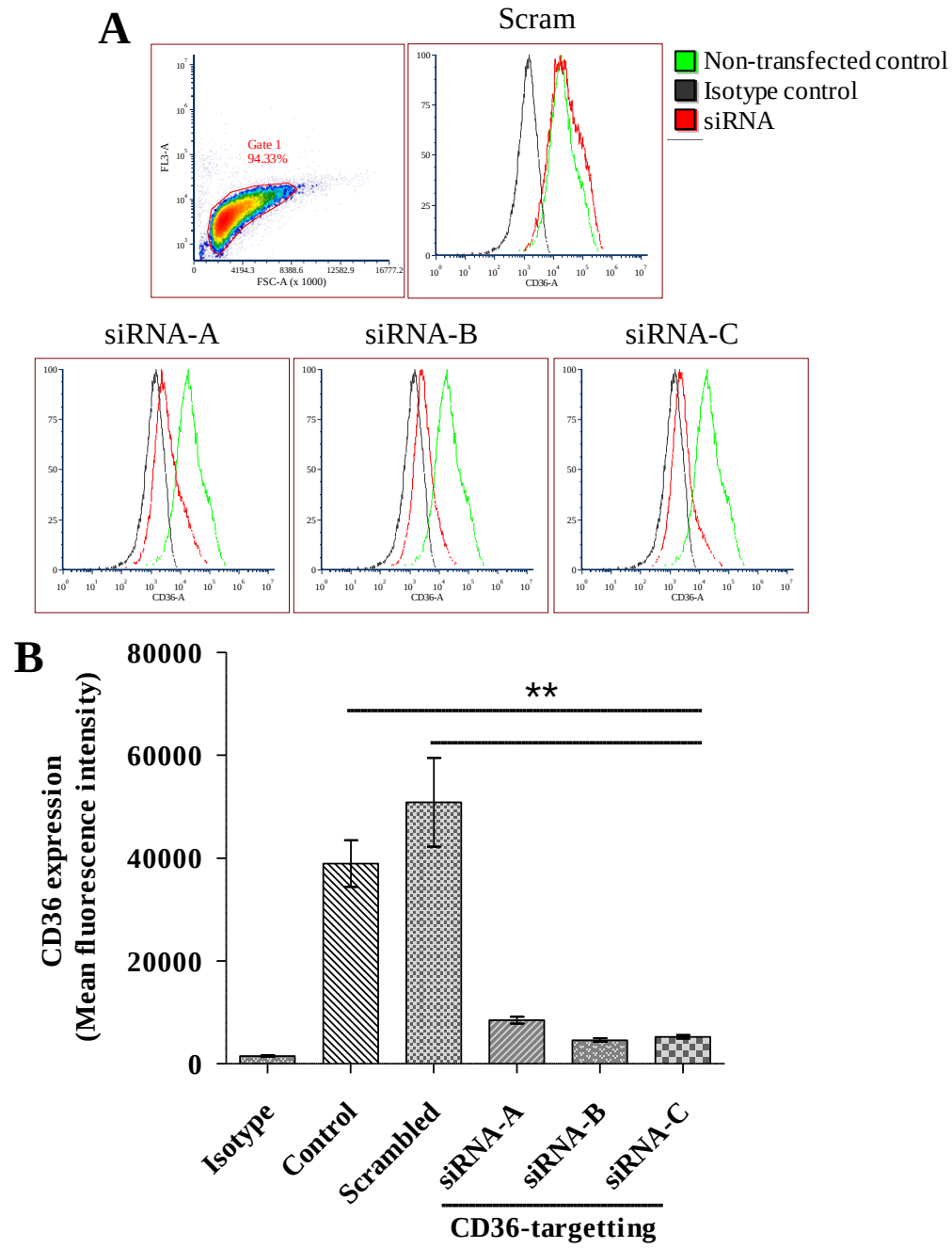


Figure 4-12. siRNA efficiently blocks cell surface protein expression of CD36 receptor in C32 cancer cell line

Cell surface expression of the CD36 receptor in C32 cancer cell line was determined by flow cytometric analysis following knockdown of CD36 via siRNA. (**A**) Representative flow cytometry scatter plot showing the gated platelet population. The population in gate 1 was assessed for expression of CD36 following transfection with scrambled (scram), siRNA-A, siRNA-B, or siRNA-C. Curves represent (green) non-transfected control cells, (black) isotype control, and (red) cells transfected with the indicated siRNA. (**B**) The mean fluorescence intensity is shown and data are expressed as mean ± SEM. Results are pooled from four separate experiments. $^{**}P<0.001$ control vs all siRNAs, one-way ANOVA with Bonferroni's post-test.

Non-transfected (control) or transfected cells (C32-CD36^{scram} or C32-CD36^{KD}) were then treated with 10nM TSP-1 for four hours, as this regimen is effective in inhibiting EC tube formation (Chu, Ramakrishnan & Silverstein 2013; Zhang et al. 2009b). As shown in Figure 4-13 (**A**-**B**), a strong trend was observed wherein TSP-1 treatment inhibited VM formation in control (non-transfected) C32 cells, although this was not significant (P=0.105). However, TSP-1 did lead to a significant inhibition of VM formation in C32-CD36^{scram} cells (P<0.05) (Figure 4-13; **C**, **D**). To further examine whether TSP-1 acts through CD36 to inhibit VM formation, C32-CD36^{KD} cells were investigated. Exposure of C32-CD36^{KD} cells to 10nM TSP-1 did not inhibit VM formation when compared to untreated C32-CD36^{KD} cells. Collectively, these studies suggest that VM formation by C32 melanoma cells can be inhibited through platelet derived TSP-1 which acts via CD36 expressed by the C32 melanoma cancer cell line.

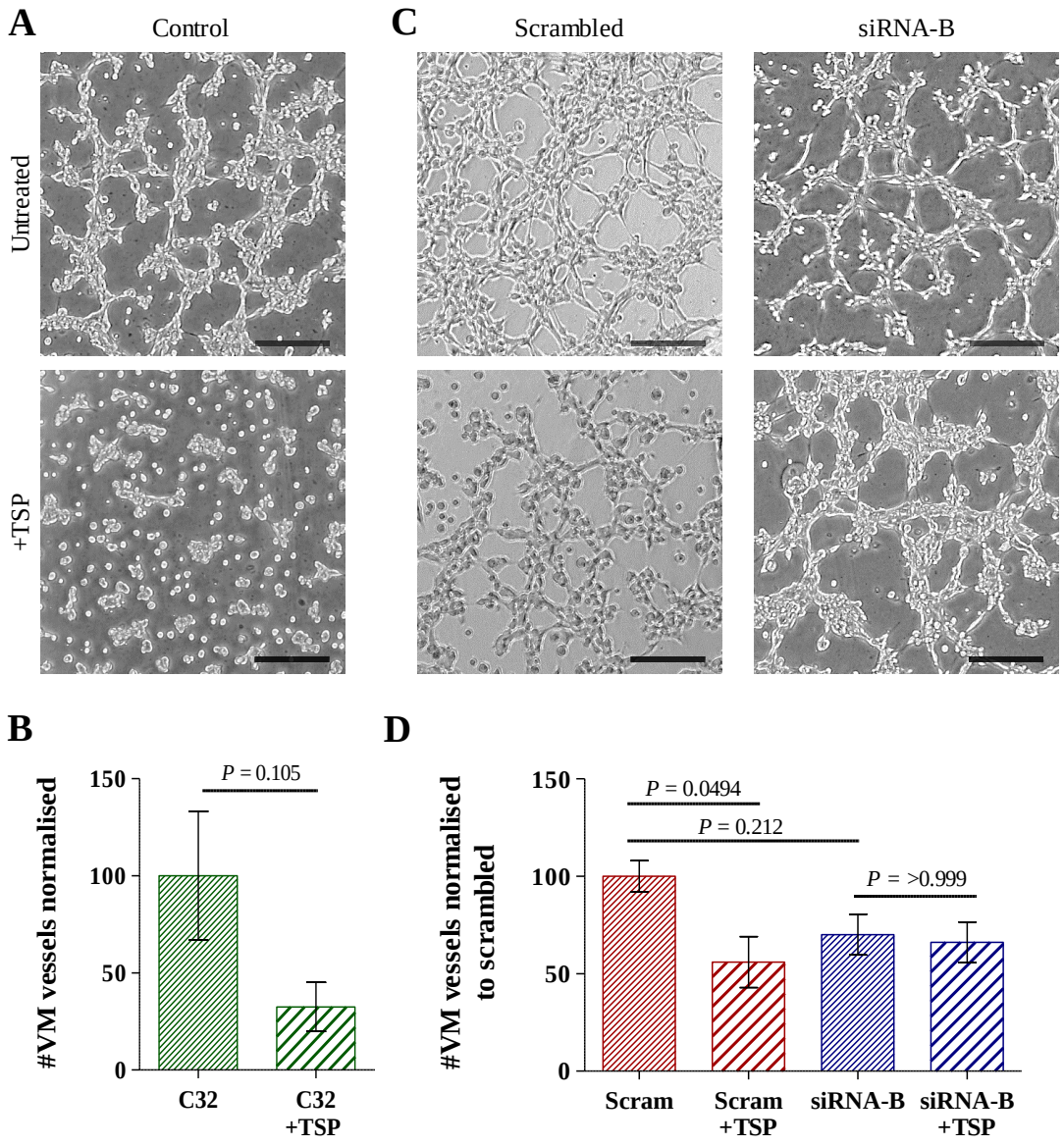


Figure 4-13. VM formation is inhibited by TSP-1 in C32 cancer cells, signalling through CD36 cell surface receptor.

(**A-B**) Representative images and quantified data of VM formation by C32 melanoma cells treated with control or 10nM TSP-1 for four hours. (**C-D**) Representative images and quantified data of VM formation by C32-scram, or C32-CD36^{KO} (siRNA-B) treated with control or 10nM TSP-1 for four hours. Data are expressed as mean ± SEM. Results are pooled from four (**A-B**) or six (**C-D**) separate experiments with 2-3 replicates within each experiment. (**B**) T-test or (**D**) one-way ANOVA with Bonferroni's post-test, **P*<0.05. Scale bar is 200µm, original magnification 40x.

4.4 **Discussion**

Results in Chapter 3 suggest that platelets inhibit VM formation in the C32 melanoma cell line, and breast cancer cell lines MDA-MB-231 and HS-578T. However, CHL-1 melanoma cells were unresponsive to platelets when assessed for VM formation. This disparity between two different melanoma cell lines provided an opportunity to critically evaluate potential mechanisms underpinning this process and became the focus of Chapter 4.

Investigations in Chapter 3 revealed that the inhibitory effect of platelets on VM formation in the C32 melanoma cell line and the HS-578T and MDA-MB-231 breast cancer cell lines is due to specific interaction between platelets and cancer cells, rather than simply spatial interference. To further examine this interaction, time lapse microscopy was performed on C32 and CHL-1 melanoma cells and this revealed a three-stage process for VM network formation. These studies found that cancer cells in the tube formation assay initiate the process as single cell structures which begin to change shape and make contact with neighbouring cells (Stage 1), before elongating and interconnecting (Stage 2), and finally forming multi-cellular inter-connecting vascular-like structures (Stage 3). The stages observed in VM formation are similar to those observed in the late stage of EC angiogenesis *in vitro* using the same or similar assay by others (Aranda & Owen 2009; Bahramsoltani et al. 2010). The presence of platelets in the assay inhibited Stage 2 of VM network formation in C32 melanoma cells, whilst CHL-1 melanoma cells proceed uninhibited to form vascular-like structures.

To investigate the possibility that surface expressed proteins on platelets and cancer cells mediate this inhibitory function, a gene expression analysis was undertaken. Integrin subunit β_3 and PSGL-1 gene expression was investigated since these markers are known to mediate adhesion of cancer cells to platelets through GPIIb/IIIa and P-selectin, respectively (Felding-Habermann et al. 1996; Kim et al. 1998a). The C32 melanoma cell line expressed very high levels of integrin *β3*, suggesting a strong adhesive potential with platelets when compared to other cancer cell lines. In contrast, CHL-1 cancer cell line expressed the lowest levels of both integrin *β3* and PSGL-1 (*SELPLG*) when compared to other cancer cell lines. Flow cytometric analysis also revealed that the protein expression level of integrin $\alpha_v\beta_3$ was high on C32 and low on CHL-1 cells, although *SELPLG* was low in both cancer cell lines. Previous studies have demonstrated that VM formation by melanoma cells *in vitro* utilises integrin $\alpha_v\beta_3$ (Vartanian et al. 2011b), and this facilitates cell-ECM contacts, cell migration and formation of cell-cell contacts. Thus, it is tempting to speculate that platelets may competitively bind to integrin $\alpha_v\beta_3$ on cancer cells and this then inhibits VM formation by preventing melanoma cell adhesion to the ECM as well as interactions between adjacent melanoma cells. The migration studies conducted herein also showed that platelets significantly inhibited the scratch wound closure assay by C32 melanoma cells but not CHL-1 melanoma cells. The gene and protein expression studies suggest that the strength of the platelet anti-VM effect may depend on the strength of interaction between platelets and cancer, i.e. strong (C32 melanoma cell line), moderate (MDA-MB-231 and HS-578T breast cancer cell lines), and weak (CHL-1 melanoma cell line).

In addition, it was also shown that α-thrombin-induced platelet releasate inhibited VM formation by the C32 melanoma cell line and the HS-578T breast cancer cell line. Notably, platelets activated with α-thrombin are known to release over 300 molecules into the supernatant (Coppinger et al. 2004), including angiogenesis inhibitors such as TSP-1.

The influence of platelets on the migration of cancer cells was also examined and it was observed that C32 melanoma cells did not migrate as far as either untreated (buffer control) cells, or CHL-1 cells co-cultured with platelets. Interestingly, the migratory ability of C32 and CHL-1 melanoma cells was not affected following exposure to tumour-conditioned platelet medium (CM). This suggests that cancer cell-induced platelet releasate differs from the releasate of α-thrombin activated platelets. Whether the cancer cell induced platelet releasate is insufficient to alter cancer cell migration or whether platelets themselves are key to cancer cell migration is yet to be determined.

Based on these observations, *in silico* analysis of genes known to regulate platelet activation and cell-cell adhesion was undertaken. The results revealed that the cancer cell lines used herein express similar and relatively low levels of *F2* (prothrombin). Whilst this suggests that cancer cells do not release inactive forms of thrombin, tissue factor (*F3*) was expressed at relatively high levels by both melanoma and breast cancer cell lines. *F3* is an activator of the intrinsic coagulation pathway which leads to thrombin generation and platelet activation (Furie & Furie 2008; Nash, Walsh & Kakkar 2001). In particular, breast cancer cell lines MDA-MB-231 and HS-578T expressed higher levels of *F3* when compared to the melanoma cell lines. High expression levels of tissue factor have previously been reported in both MDA-MB-231 and HS-578T breast cancer cell lines (Kirschmann et al. 1999; Zhang et al. 2011). Together with the work by others, these

findings indicate that cancer cells can activate the coagulation pathway, generating thrombin which can lead to platelet activation and release of contents.

Platelets can release various anti-angiogenic molecules including angiostatin, endostatin and TSP-1, along with pro-angiogenic factors such as VEGF, PDGF and bFGF (Coppinger & Maguire 2007; Gear & Camerini 2003; Peterson et al. 2010; Whiteheart 2011). The anti-VM effect of supernatants from activated platelets suggests that the cancer cells respond to anti-angiogenic compounds released by platelets. Gene expression analysis revealed high levels of both *NCL* (nucleolin, an endostatin receptor) and *ANAX2* (annexin II, an angiostatin receptor), across all melanoma and breast cancer cell lines in the database, with no detectable differences. Since *NCL* is expressed highly across all cancer cell lines, this may suggest that the cancer cell specific anti-VM effect may not be dependent on endostatin release. In support of this, studies by others have shown that endostatin is ineffective at inhibiting VM formation by human metastatic (uveal and cutaneous) melanoma cells *in vitro* (van der Schaft et al. 2004), and in glioblastoma (Liu et al. 2011b). In contrast, angiostatin is known to bind to integrin $\alpha_v\beta_3$, acting as an antagonist to inhibit the binding of other RGD sequence-containing ligands (Tarui, Miles & Takada 2001). In melanoma, an integrin $\alpha_v\beta_3$ blocking antibody inhibited VM formation *in vitro* (Vartanian et al. 2011b), and since the cancer cells differ in their expression of integrin *β3* subunit, angiostatin may regulate the cancer-dependent anti-VM effect mediated by platelets. The effectiveness of angiostatin in VM formation is unknown and the significance of this observation remains to be elucidated.

Gene expression studies also identified high expression of *CD36* (TSP-1 receptor) in the C32 melanoma cell line, a finding that was confirmed at the protein level by flow cytometric analysis. This supports a previous report by Silverstein and co-workers who

documented the C32 melanoma cell line expressing high levels of CD36 at the cell surface (Silverstein et al. 1992). Conversely, *CD36* was expressed at very low (negligible) levels in CHL-1 melanoma and MDA-MB-231 and HS-578T breast cancer cell lines. This was further supported by flow cytometric analysis of surface protein levels. The ligand for CD36 receptor, TSP-1, is amongst the most abundant proteins contained in platelets (Burkhart et al. 2012) making up as much as 20% of the total released contents (Baenziger, Brodie & Majerus 1972). Studies by Silverstein and colleagues showed that C32 cancer cells strongly bind to TSP-1 via CD36 in a 1:1 ratio *in vitro*, and this is inhibited when CD36 protein expression is reduced by siRNA silencing (Silverstein et al. 1992). This suggests that the high expression of CD36 receptor by C32 melanoma cells may be responsible for the high sensitivity to the anti-VM effect of platelets in comparison to other cancer cell lines. Gene expression analysis further revealed high expression of *KDR* (VEGFR2) in C32 melanoma cells when compared to the other cancer cell lines. As discussed previously, VEGFR2 mediates pro-angiogenic effects in ECs following VEGF binding. In the presence of TSP-1 binding to CD36, VEGFR2 is dephosphorylated leading to inhibited EC tube formation (Chu, Ramakrishnan & Silverstein 2013). Whilst further validation studies were not performed here, others have demonstrated through microarray analysis and immunoblot that the C32 melanoma cell line strongly expresses both VEGFR2 mRNA and protein levels (Chen et al. 2015). Taken together, these results suggest that activation of the CD36/TSP pathway in C32 melanoma cell line may be a mechanism through which VM formation is attenuated.

To investigate the involvement of the TSP-1/CD36 pathway, VM tube formation assays with C32 melanoma cells were undertaken with recombinant TSP-1 and knockdown of CD36 via siRNA. An unexpected observation was that knockdown of CD36 receptor alone showed reduced VM formation when compared to control cells treated with a

scrambled siRNA, although this was not statistically significant (but repeatable). This effect may be due to inhibition of cancer cell binding to the ECM since CD36 is also a receptor for collagen, thus promoting adhesion (Diaz-Ricart et al. 1993; Tandon, Kralisz & Jamieson 1989). Furthermore, CD36 is also known to co-precipitate with integrin β_3 (Miao et al. 2001), a subunit of integrin $\alpha_v\beta_3$. Since integrin $\alpha_v\beta_3$ is important in melanoma VM formation (Vartanian et al. 2011b) and is highly expressed in C32 melanoma cells, eliminating a protein-protein interaction by knocking down CD36 may also inhibit VM formation. Indeed, CD36 is a known receptor for collagen type-1 which may assist tumour cells to bind to the ECM (Tandon, Kralisz & Jamieson 1989; Uray, Liang & Hyder 2004), and, in turn, may facilitate VM formation (Seftor et al. 2012). Therefore, CD36 itself may be important in supporting VM formation, although further studies are necessary to determine the significance of these findings and elucidate the dual role CD36 may have in malignancy. In addition to its potential involvement in cell adhesion, there are other known roles of CD36, in particular its participation in fat metabolism recognising a number of lipid ligands, native and oxidised lipoproteins (Pepino et al. 2014). CD36 is involved in lipid uptake from the extracellular environment, mediates lipid β-oxidation (Kennedy & Kashyap 2011) resulting in ATP production in cells (Coburn et al. 2000; Ibrahimi et al. 1999). In a recent publication by Pascual and colleagues, oral squamous cell carcinomas expressing mutant CD36 demonstrated caspase-3 immunoreactivity (Pascual et al. 2017). It was hypothesised that loss of CD36 leads to continuous lipid accumulation and eventual lipotoxicity resulting in cell death. While the current work in this Chapter has been carried out *in vitro* and is therefore not likely to be impacted by lipid content in the extracellular environment, the function of CD36 in fat metabolism should be considered in future prospective *in vivo* studies investigating the role of CD36

in VM. Currently, there are no reports which have investigated the role of CD36 in VM, and the findings in this Chapter are the first to be documented.

Treatment with TSP-1 inhibited VM formation in the CD36-expressing C32 melanoma cells, suggesting that TSP-1 may mediate an anti-VM response. Knockdown of CD36 also inhibited the anti-VM effect of TSP-1 in C32 cells and, as similar VM capacity was observed in TSP-1-treated and untreated C32-CD36^{KD} cells, this suggests that TSP-1 mediates anti-VM effects via CD36. This finding is similar to the effects of TSP-1 on EC tube formation observed by others (Chu, Ramakrishnan & Silverstein 2013; Dawson et al. 1997). Studies by Dawson and colleagues showed that when CD36-negative HUVECs were transfected with plasmid to overexpress CD36 protein, cells became sensitive to the effects of TSP-1, leading to reduced tube formation and migration (Dawson et al. 1997). In more recent studies, TSP-1 binding to CD36 has been shown to inhibit VEGF/VEGFR2-mediated tube formation by microvascular ECs via recruiting SHP-1, which dephosphorylates VEGFR2 (Chu, Ramakrishnan & Silverstein 2013). VM tube formation by the C32 melanoma cell line may occur through similar signalling pathways in that TSP-1 inhibits VM formation by cancer cells which express CD36.

Additional surface proteins may also be involved in regulating VM. Elevated expression of CD47, another TSP-1 receptor, has been observed in patients with ovarian, breast, colon, bladder, hepatocellular and prostate cancer as well as glioblastoma (Willingham et al. 2012). Gene expression analysis in this Chapter found elevated expression of CD47 in the cancer cell lines in which exposure to platelets inhibited VM-formation (i.e. C32, MDA-MB-231 and HS-578T cells). The high sensitivity of C32 melanoma cells towards reduced VM in response to platelets and TSP-1 may also be related to high expression of CD47. Likewise, the negative effect of platelets on VM by MDA-MB-231 and HS-578T may be less reliant on CD36, but more reliant on their elevated levels of CD47 gene

expression. Notably, CHL-1 melanoma cells express negligible levels of both CD36 and CD47 and were completely resistant to the anti-VM effects of platelets. In very recent studies, the knockdown of CD47 promoted tumour growth and angiogenesis whilst elevating VEGF and VEGFR2 expression in a murine model of prostate cancer (Gao et al. 2017). From these studies, it is tempting to speculate that the anti-VM effects of platelets are mediated through TSP-1 and may be most prevalent in cancer cells with high CD36 and/or CD47 expression.

High TSP-1 expression is associated with improved survival in patients with melanoma, ovarian, and non-small cell lung cancer (Alvarez et al. 2001; Straume & Akslen 2005; Yamaguchi et al. 2002). Conversely, lower TSP-1 expression is associated with elevated risk of recurrence and poorer survival in patients with breast, pancreatic, and bladder cancer (Grossfeld et al. 1997; Ioachim et al. 2012; Jenkinson et al. 2016). Interestingly, a pro-tumorigenic role for TSP-1 in malignancy has also been described, in which elevated expression of TSP-1 was associated with a significant increase in disease recurrence in breast cancer patients (Fontana et al. 2005) and decreased survival in melanoma patients (Straume & Akslen 2001). These contradictory observations may be explained through a proposed dual role for TSP-1 in angiogenesis, wherein fragments generated by cleavage of TSP-1 promote FGF-2-induced angiogenesis (25-kDa) or inhibit the pro-angiogenic effects of FGF-2 (140-kDa) *in vivo* (Taraboletti et al. 2000). These effects may possibly depend on expression of TSP-1 receptors on tumour and/or stromal cells (Clezardin et al. 1993).

In recent studies, a loss of CD36 expression has been shown to potentiate the aggressiveness of breast cancer (DeFilippis et al. 2012). CD36 expression was absent in tumour tissue compared to normal adjacent tissue, and low levels of expression were also

associated with high mammographic density (MD) (a strong risk factor for invasive breast cancer compared to low MD) (DeFilippis et al. 2012). Similar effects were also observed in colon cancer, wherein elevated stromal expression of CD36 correlated with decreased vascularisation and improved prognosis in comparison to absence of CD36 expression (Tsuchida et al. 1999). The expression of CD36 also correlates with poor prognosis (disease-free survival and overall survival) in luminal A breast cancer, lung squamous cell carcinoma, and bladder cancer (Pascual et al. 2017), suggesting a dual role for CD36 in malignancy as reported for TSP-1 (Fontana et al. 2005; Straume & Akslen 2001). Similarly, high mRNA expression of CD47 correlated with decreased progression-free survival of ovarian cancer and reduced overall survival of glioma patients (Willingham et al. 2012). Elevated protein expression of CD47 has also been correlated with clinical staging, lymph node metastasis and distant metastasis in non-small cell lung cancer patients (Zhao et al. 2016). Whilst TSP-1 signalling *in vitro* and *in vivo* can mediate inhibitory effects on malignancy, the above studies underline the multifunctional role of both CD36 and CD47 expression, which may depend on alternate ligand-specific interactions (e.g. ox-LDL and SIRPα, respectively).

The results presented in this Chapter suggest that the anti-angiogenic and anti-tumorigenic properties of platelets that operate through their TSP-1 content (Zaslavsky et al. 2010), may also be effective at blocking VM network formation by highly malignant cancer cells. TSP-1/CD36 has been identified as a novel mechanistic pathway which can be used to therapeutically target VM formation in cancer. Thus therapies which can regulate both TSP-1 and CD36 expression and improve prognosis in cancer patients (DeFilippis et al. 2012), may also be utilised to potentially inhibit VM formation.

4.5 Summary

The studies described in this Chapter, in conjunction with the studies in Chapter 3, have revealed that VM formation *in vitro* is inhibited by platelets through both direct interaction and platelet-released factors. This effect on VM was associated with the expression of specific adhesion molecules by both platelets and cancer cells, and the expression of receptors to anti-angiogenic factors released by platelets. A novel TSP-1/CD36 pathway for the regulation of VM formation in cancer cells and mediated through the contents released by platelets has been identified. A caveat of the results presented thus far is that all experiments have been conducted using in vitro assays which may not precisely mirror the complex biology of whole organisms. Accordingly, the role of platelets on VM formation *in vivo* will be the focus of the next Chapter.

Chapter 5. Investigating the role of platelets in vasculogenic mimicry in breast cancer *in vivo*

5.1 Introduction

5.1.1 Breast cancer

In Australia, breast cancer is the most common cancer in women and represents approximately 28% of all cancers diagnosed in women. About 15,000 women are diagnosed each year, and one in eight will be diagnosed by the age of 85 (Cancer Council).

Breast cancer is categorised into distinct clinical subtypes based on the location of the originating cell type. These include basal (arising from the basal/myoepithelial cells) or luminal breast cancer (arising from the luminal cells) (Figure 5-1). These are further separated into four categories based on expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Onitilo et al. 2009; Zaha 2014). Luminal breast cancer is further subdivided into Luminal-A and Luminal-B ($HER2^-$ or $HER2^+$), HER2 enriched, and basal-like/triple negative breast cancer (TNBC) when none of these hormone receptors are expressed (Table 5-1). TNBC can be further classified into four molecular subtypes (by their gene expression profile) with different biological characteristics, including basal-like (BL1 and BL2), mesenchymal (M) and luminal androgen receptor (LAR) type (Lehmann et al. 2011; Lehmann et al. 2016). More specifically, the BL1 signature includes high expression of genes involved in cell cycle and DNA damage, while the BL2 subtype is characterised by expression of markers pertaining to myoepithelial and growth factor signalling. Subtype M has elevated expression of genes involved in epithelial-to-mesenchymal transition (EMT) and growth factor signalling, while the LAR subtype is driven by activation of its androgen receptor and is characterised by expression of luminal genes (Lehmann et al. 2011; Lehmann et al. 2016). Studies using the Prosigna Breast

Cancer Prognostic Gene Signature Assay (formerly called the PAM50 test) have shown that BL1 subtype patients have the best overall survival (OS) compared with other subtypes, benefitting the most from neoadjuvant chemotherapy (Lehmann et al. 2016).

In current practice, patients who express hormone receptors (ER/PR+) receive endocrine therapy such as tamoxifen or aromatase inhibitors, while trastuzumab, a monoclonal antibody, is used to treat patients with $HER2^{+}$ cancer (Goldhirsch et al. 2013). Since TNBC cells lack expression of HER2 and both hormone receptors, endocrine and anti-HER2 therapy are not effective. In comparison to other breast cancer subtypes, patients with TNBC have a very poor prognosis (Dent et al. 2007; Linderholm et al. 2009; Zhang et al. 2014a). Notably, when compared to non-TNBC, patients with TNBC have a significantly higher incidence of lymph metastases (77.3% TNBC versus 69.8% non-TNBC) and higher histological grade II/III tumours (97.1% TNBC versus 91.2% non-TNBC) (Agarwal et al. 2016). However, TNBC patients respond significantly better as determined by a pathologic complete response (pCR) to neoadjuvant chemotherapy such as anthracycline and taxane combination when compared to non-TNBC patients (22-27.5% TNBC versus 11-17.1% non-TNBC) (Agarwal et al. 2016; Liedtke et al. 2008). Despite this, TNBC patients with residual disease had a poorer OS (Liedtke et al. 2008). While stage III TNBC patients had reduced OS (~81.8 months for TNBC versus ~97.90 months for non-TNBC) and disease-free survival (DFS; ~89.2 months for TNBC versus ~113.8 months for non-TNBC) in comparison to non-TNBC patients, this difference was not observed in patients with stages I, II and IV cancer (Agarwal et al. 2016).

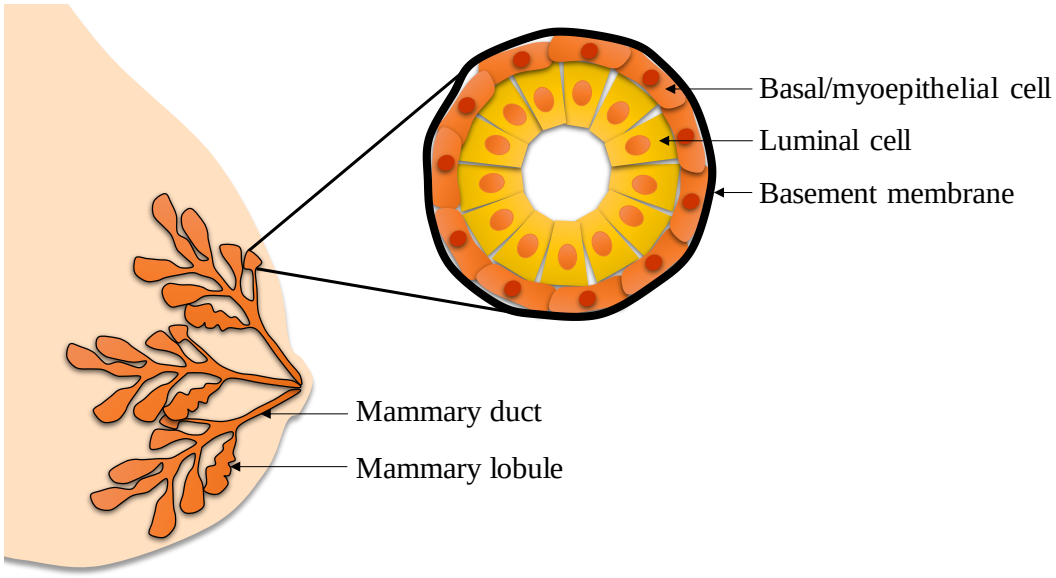


Figure 5-1. The structure of the human breast glandular tissue and ducts

The human breast is composed of glandular tissue, fatty tissue, and stroma. The glandular tissue is composed of lobules and ducts. The duct in the terminal lobular unit is composed of an inner lobular luminal cell layer and an outer layer of myoepithelial cells.

Table 5-1. Immunohistochemical classification of breast cancer

Subtype	ER expression	PR expression	HER2 expression
Luminal A	+	+	+
Luminal B (HER2-)	+	low or -	-
Luminal B (HER2+)	+	±	+
HER2 enriched	-	-	++
Basal-like/triple negative	-	-	-

ER; estrogen receptor, **PR**; progesterone receptor, **HER2**; human epithelial receptor type 2.

Chemotherapy drugs used in TNBC include anthracyclines (e.g. doxorubicin, epirubicin), taxanes (e.g. paclitaxel, docetaxel), antimetabolites (e.g. capecitabine, gemcitabine), and platinum agents (e.g. cisplatin, carboplatin) (Joensuu & Gligorov 2012; Wahba & El-Hadaad 2015). In 2009, Linderholm and colleagues reported that VEGF protein levels were significantly elevated in the tumours excised from patients with TNBC compared with non-TNBC (Linderholm et al. 2009). Because of this, an angiogenesis inhibitor (bevacizumab, monoclonal VEGF blocking antibody) was trialled and reached Phase II-III clinical trials, as summarised in Table 5-2, but for reasons detailed below, this treatment option was discontinued. For TNBC patients, a significantly higher pCR rate with bevacizumab was observed in both the GeparQuinto (39.3% with bevacizumab versus 27.9% without) and CALGB trials (59% with bevacizumab versus 48% without). In contrast, the NSABP B-40 trial observed that bevacizumab was more effective in hormone receptor positive (HR+) patients (i.e. ER^+PR^-, or ER^-PR^+, or ER^+PR^+) than in TNBC patients (see Table 5-2). The administration of bevacizumab demonstrated no improvement in OS of TNBC patients in both GeparQuinto (P=0.891) and S0800 trials (P=0.14). In contrast to this, significantly improved OS was observed in all HER2 breast cancers (P=0.04), particularly in the HR^+ subgroups in the B-40 trial. In all the trials, bevacizumab resulted in adverse events (AE) of grade 2 (moderate AE), grade 3 (severe AE), or grade 4 (life-threatening or disabling AE).

Table 5-2. Neoadjuvant chemotherapy trials with bevacizumab in triple-negative breast cancer (TNBC)

Study name	Chemotherapy regimen	Bev dose	non-TNBC pCR	TNBC pCR	Ref.
CALGB, Phase II, NCT00861705	wP→ddAC + Bev vs wP→ddAC; wPCb→ddAC + Bev vs wPCb→ddAC	10 mg/kg iv	Not investigated	59% with Bev vs 48% without (*P*=0.0089)	(Sikov et al. 2015)
S0800, Phase II, NCT00856492	wP→ddAC + Bev vs wP→ddAC; ddAC→wP+Bev vs ddAC→wP	10 mg/kg iv	24% with Bev vs. 18% without (*P*=0.41)	59% with Bev vs. 29% without (*P*=0.014)	(Nahleh et al. 2016)
GeparQuinto, Phase III, NCT00567554	EC→T+Bev vs EC→T	15 mg/kg iv	7.7% with Bev vs. 7.8% without (*P*=1.0)	39.3% with Bev vs 27.9% without (*P*=0.003)	(von Minckwitz et al. 2012; von Minckwitz et al. 2014)
NSABP B-40, Phase III, NCT00408408	T→AC+Bev vs T→AC; TX→AC+Bev vs TX→AC; TG→AC+Bev vs TG→AC	15 mg/kg iv	23.2% with Bev vs. 15.1% without (*P*=0.007)	52% with Bev vs 47% without (*P*=0.34)	(Bear et al. 2015; Bear et al. 2012)

wP, weekly paclitaxel; **ddAC**, wP followed by doxorubicin and cyclophosphamide once every 2 weeks; **A**, doxorubicin; **Bev**, Bevacizumab; **C**, cyclophosphamide; **Cb**, carboplatin; **E**, epirubicin; **G**, gemcitabine; **pCR**, pathologic complete response; **T**, docetaxel; **TNBC**, triple-negative breast cancer; **X**, capecitabine.

The failure of bevacizumab to improve OS, conflicting pCR rates in phase II-III clinical trials, and the considerable toxicity of bevacizumab, quickly diminished hopes for its use as a treatment option for TNBC. The unremarkable response towards bevacizumab may be due to the highly heterogeneous nature of TNBC, and intimating that alternative mechanisms to angiogenesis may also be supporting TNBC progression (Lenzer 2011). It has been suggested that vasculogenic mimicry (VM), documented to be unresponsive to angiogenic inhibitors (van der Schaft et al. 2004), may be one of the mechanisms driving TNBC. Indeed, whilst VM is observed in both TNBC and non-TNBC breast cancer (Buijs et al. 2004; Liu et al. 2013b; Shirakawa et al. 2002b), it is more frequently found in patients with TNBC (35.5% of TNBC versus 17.8% of non-TNBC) (Zhang et al. 2014a). More importantly, the presence of high levels of VM content in TNBC patient samples is associated with poor survival (Plantamura et al. 2014). In breast cancer patients, VM is also commonly associated with shorter DFS and OS (Buijs et al. 2004) as well as higher percent of haematogenous recurrence (P=0.059) (Shirakawa et al. 2002b).

5.1.2 **Vasculogenic mimicry and gene expression profile**

Vasculogenic mimicry is demonstrated by staining sections of tumour samples for CD31 to identify EC-lined vessels and with periodic acid-Schiff (PAS), which highlights polysaccharides in the basement membrane of VM structures (Maniotis et al. 1999). This approach allows VM structures to be identified at PAS+ CD31-, and clearly discriminated from the CD31+ EC-lined blood vessels. While initially identified in aggressive uveal melanoma (Maniotis et al. 1999), VM has also been found to occur in over 11 different human cancers (Yang et al. 2016) as well as xenograft models of TNBC breast cancer (Plantamura et al. 2014; Shirakawa et al. 2002b; Sun et al. 2017; Zhang et al. 2014a). Recent studies using xenograft models of TNBC observed elevated protein expression of

vascular endothelial (VE)-cadherin and Twist1 (an EMT marker) in VM-positive compared to VM-negative non-TNBC xenografts (Sun et al. 2017).

Studies have shown that during VM development a range of genes involved in a variety of functions are upregulated, including remodelling of the extracellular matrix, cell-surface glycoprotein expression, cell-cell adhesion, tumour cell invasion, basement membrane development and tyrosine kinase activity (Hendrix et al. 2003a; Seftor et al. 2001). Current genes deemed to define a VM signature/profile across various malignancies include VE-cadherin (*CDH5*), epithelial cell kinase (EphA2), Laminin-γ2-chain (LAMC2), VEGF receptor 2 (KDR), and matrix metalloproteinases (MMPs, including MMP1, MMP2, MMP9 and MMP14) (Francescone et al. 2012; Liu et al. 2013a; Liu et al. 2013b; Seftor et al. 2002b; Seftor et al. 2002c; Seftor et al. 2001; Yao et al. 2013). Interaction of EphA2 with VE-cadherin, both of which are co-expressed on the surface of highly malignant cells, leads to activation of the phosphoinositide-3-kinase (PI3K) pathway in VM (Hess et al. 2003). This results in activation of MMP2 and MMP14, which in turn cleave laminin-γ2 chain. In breast epithelial cells, MMP2 cleaves laminin-γ2 chain into pro-migratory γ2' and γ2x fragments (Giannelli et al. 1997). These pro-migratory fragments were found to be essential in VM induction in poorly aggressive melanoma cell lines treated with aggressive tumour cell-preconditioned matrix *in vitro* (Seftor et al. 2002c). In a panel of breast cancer cell lines (including MDA-MB-231 cells), MMP14 can also cleave laminin γ2 chain, having a direct effect on cancer cell migration (Koshikawa et al. 2000).

5.1.3 **Aspirin as a modulator of platelet function and breast cancer progression**

Aspirin is a commonly used non-steroidal anti-inflammatory drug (NSAID) for the prevention of clotting and treatment of pain, fever, and inflammation (Vane 1971; Vane & Botting 2003). Aspirin mediates its action by acetylating the active site of the cyclooxygenase (COX) enzymes, COX-1 (Serine 530) and COX-2 (Serine 516) (Loll, Picot & Garavito 1995; Lucido et al. 2016). This in turn prevents the binding of arachidonic acid (AA), and consequently blocks prostaglandin synthesis and production of thromboxane A_2 (TXA_2, a platelet agonist). While aspirin can inhibit the function of both COX-1 and COX-2, it is known to be more specific for COX-1, being effective at low doses (<100mg daily) without blocking COX-2 function (Blanco et al. 1999; Dovizio et al. 2013a; Ornelas et al. 2017; Patrignani, Filabozzi & Patrono 1982). Inhibiting COX-1 in platelets blocks TXA_2 formation and consequently reduces platelet aggregation. At high doses, aspirin inhibits COX-1 as well as COX-2, leading to inhibitory effects on platelet aggregation as well as both analgesic and anti-inflammatory effects, respectively (Ornelas et al. 2017). As COX-1 is also expressed by ECs, aspirin inhibits its function in the blood vessel wall leading to reduction of antithrombotic PGI_2 production (Caughey et al. 2001; Warner, Nylander & Whatling 2011)

Studies of VM in breast cancer have reported the involvement of COX-2 (Basu et al. 2006; Robertson et al. 2010). Basu and colleagues demonstrated that gene knockdown of COX-2 in MDA-MB-231 cells *in vitro* significantly inhibited VM formation, which could then be restored with exogenous PGE_2 (Basu et al. 2006). It is known that PGE_2 promotes migration and invasion of various cancer cells (Menter et al. 2014), including murine C3L5 mammary carcinoma cells and human MDA-MB-231 breast cancer cells (Timoshenko et al. 2003). Platelets also release PGE_2 as well as TXA_2, both being synthesised from AA by COX-1 which is constitutively expressed in platelets (Guillem-

Llobat et al. 2016). They are known to also express COX-2 which synthesises PGE_2 from AA (Cipollone, Rocca & Patrono 2004). *In vitro*, PGE_2 and TXA_2 formation in platelets can be inhibited with aspirin via its suppression of COX-1 activity (Dovizio et al. 2013b). This inhibitory effect also occurs in healthy humans where low doses of aspirin (100mg daily) almost completely inhibit synthesis of TXA_2 (Patrignani, Filabozzi & Patrono 1982).

It has been recognised for some time that aspirin use has the potential to reduce the risk of a variety of cancers, including breast cancer (Rothwell et al. 2012a; Rothwell et al. 2010; Rothwell et al. 2012b), although the benefit with the latter may be related to the breast cancer subtype (Marshall et al. 2005; Ready et al. 2008; Zhang et al. 2008). In a prospective observational study, which did not stratify cohorts based on hormone receptor or HER2 status, regular aspirin use in breast cancer patients resulted in reduced risk (43%) of distant recurrence, as well as reduced risk of mortality (74%) compared with non-use (Holmes et al. 2010). Other studies found no significant reduction in breast cancer incidence (Jacobs et al. 2007), even following HR status stratification (Gill et al. 2007; Zhang et al. 2008). In contrast, Marshall and colleagues observed increased risk of HR^- (ER^-PR^-) breast cancer in comparison to non-regular aspirin users, and no effect on HR^+ (ER^+PR^+) breast cancer (Marshall et al. 2005). Others have observed reduced risk of ER^+ breast cancer but not in ER^- breast cancer with aspirin use compared with non-use (Gierach et al. 2008). Finally, in Stage II and III TNBC patients, very recent studies found that aspirin use improves DFS and reduces the risk of distant metastases (Shiao et al. 2017). Although Shiao and colleagues present evidence that aspirin improves outcome in TNBC (Shiao et al. 2017), further work is necessary to address the earlier conflicting studies discussed above.

The use of aspirin has also shown promising results in animal models of breast cancer, although studies are limited (Kuznetsov et al. 2012; Maity et al. 2015). For example, administration of aspirin (75mg/kg, equivalent to 360mg human dose) in TNBC MDA-MB-231 xenograft models found that aspirin elicits both preventative and therapeutic effects, preventing tumour growth and inducing caspase-3-mediated apoptosis in the tumour (Maity et al. 2015). This study by Maity and colleagues did not investigate whether aspirin had effects on tumour perfusion (i.e. EC-lined or VM structures) (Maity et al. 2015). While the benefits of low-dose aspirin (10-25mg/kg, equivalent to ≤150mg human dose) in animal models of neuroblastoma and colorectal cancer have been reported (Carlson et al. 2013; Guillem-Llobat et al. 2016; Lichtenberger et al. 2017; Reuter, Zhang & Miller 2002), the effects of low-dose aspirin in breast cancer are yet to be elucidated.

5.1.4 **Platelets and breast cancer**

A direct association between platelet count and breast cancer has been documented (Stravodimou & Voutsadakis 2013; Taucher et al. 2003). For example, in a large retrospective multivariate analysis of 4,300 patients with early-stage breast cancer, 161 patients (3.7%) with thrombocytosis (>453x10^9/L) had significantly lower OS compared to patients without thrombocytosis (<453x10^9/L) (OS, 71.0 versus 99.5 months, *P*=0.0064) (Taucher et al. 2003). Similar observations have been made in metastatic breast cancer where patients with thrombocytosis (>350x10^9/L) had lower OS (*P*=0.038) and lower progression free survival (*P*=0.008) (Stravodimou & Voutsadakis 2013). This suggests that platelets may contribute to a negative outcome in breast cancer patients.

As discussed previously, platelets contain several proangiogenic growth factors within their alpha granules including VEGF, PDGF, FGF, angiopoietin-1/-2, and EGF (Coppinger et al. 2004), and have been shown to stabilise tumour angiogenic vessels and

maintain the vascular integrity in mouse models of breast cancer, Lewis lung carcinoma, and B16/F10 melanoma (Demers et al. 2011; Ho-Tin-Noe et al. 2008). Depletion of platelets also inhibits growth in models of ovarian cancer (Yuan & Liu 2015). Stone and colleagues demonstrated that in addition to reducing tumour growth, platelet depletion also resulted in reduced tumour vessel density and elevated apoptosis in syngeneic models of ovarian cancer (Stone et al. 2012). Reduced tumour volume was observed in platelet-deficient mice with 4T1 TNBC xenografts compared with WT mice (Coupland, Chong & Parish 2012). Additionally, using the same model Coupland and colleagues detected lower lung metastases in platelet-depleted mice which occurred independently of natural killer (NK) cells (Coupland, Chong & Parish 2012), despite earlier studies which demonstrated that platelets protect cancer cells from NK-cell attack in circulation (Nieswandt et al. 1999; Palumbo et al. 2005). While more recent studies report no change in tumour volume following platelet depletion in the syngeneic 4T1 breast cancer model, reduced tumour vessel density and lung metastases was observed in the B16 syngeneic melanoma model (Li et al. 2014a). This was associated with reduced levels of circulating VEGF and the endothelial expressed marker, VE-cadherin.

Angiogenesis inhibitors (anginex, TNP-470 and endostatin) which prevent EC-mediated tube formation have failed to inhibit VM formation *in vitro* (van der Schaft et al. 2004). Furthermore, recent studies show that inhibiting tumour angiogenesis promotes VM formation *in vivo* (Schnegg et al. 2015; Sun et al. 2017; Xu et al. 2012). For example, in the SKOV3 xenograft model of ovarian cancer, short-term anti-angiogenic treatment (bevacizumab) promoted VM as well as increased metastases and resulted in marked elevation of hypoxia-inducible transcription factor-1α (HIF-1α) protein, a key driver of the hypoxic response (Xu et al. 2012). Similarly, studies in TNBC xenograft models using breast cancer MDA-MB-231 and HS-578T cell lines, have shown that short-term

treatment with another angiogenesis inhibitor, sunitinib (a tyrosine kinase inhibitor which targets VEGFR, PDGFR and FGFR), elevates hypoxia and VM formation whilst attenuating tumour vascularisation and tumour growth (Sun et al. 2017). Importantly, HIF-1α is a potent initiator of VM formation in melanoma cells (Kirschmann et al. 2012; Sun et al. 2007; Zhang et al. 2009a). In breast cancer cells *in vitro*, HIF-1α has been associated with a more aggressive phenotype (Helczynska et al. 2003) and an elevation in the cancer stem cell (CSC) population has been observed in TNBC xenografts (Conley et al. 2012). This is significant as CSCs have been shown to promote VM formation (Liu et al. 2013b; Yao, Ping & Bian 2011). It has been proposed that by inhibiting angiogenesis, tumours may respond to hypoxia-induced HIF-1α upregulation which in turn promotes VM as a mechanism to restore tumour perfusion (Schnegg et al. 2015).

Platelets are shown to be an important source of angiogenic compounds, and either platelet depletion (antibody mediated) or inhibition of platelet function (i.e. with aspirin at low doses ≤25mg/kg) results in reduced tumour angiogenesis, growth and metastasis *in vivo* (Guillem-Llobat et al. 2016; Labelle, Begum & Hynes 2014; Li et al. 2014a; Lonsdorf et al. 2012; Palumbo et al. 2005). While the evidence demonstrating their role in tumour growth and angiogenesis is increasing, the role of platelets in VM formation *in vivo* is currently unknown. Notably, short-term treatment with inhibitors of angiogenesis (e.g. bevacizumab, and sunitinib) can promote VM formation in TNBC xenografts (Sun et al. 2017; Xu et al. 2012; Zhang et al. 2014a), whilst inhibiting tumour growth. Based on evidence in the literature, it is hypothesised that inhibiting the pro-angiogenic function of platelets with aspirin may lead to promotion of VM formation *in vivo*. The focus of this Chapter is to investigate the contribution of platelets to VM formation *in vivo*. To achieve this, an orthotopic xenograft model of breast cancer with TNBC MDA-MB-231-

LM2 cells is used in mice where platelets are inactivated by the administration of daily low-dose aspirin (25mg/kg).

5.2 Materials and methods

5.2.1 Xenograft model of triple negative breast cancer model

Materials and methods for the *in vivo* study have been described in detail in Chapter 2 (section 2.6). A total of 22 female NOD/SCID mice (6-8 weeks of age), which were tested over two independent experiments, were included in the animal study (Figure 5-2). Mice were injected subcutaneously into the mammary fat-pad (MFP) with $1x10^6$ cells of a firefly luciferase-tagged breast cancer cell line, MDA-MB-231 LM2 (231-LM2). The MDA-MB-231 cell line is a mesenchymal type/basal-type TNBC cell line and 231-LM2 indicates that it has been transformed to stably expresses firefly luciferase (Minn et al. 2005).

5.2.1.1 *Bioluminescence imaging and other parameters*

On day one after tumour cell injection, and weekly thereafter (days 8, 15, 22, and 29 post implantation) D-luciferin was injected intra-peritoneally (3mg/mouse) 10 min prior to bioluminescence imaging using the Xenogen IVIS 100 imaging system (Li et al. 2014b). Photon emission (Flux) was quantified using Living Image Software (Xenogen; Perkin Elmer, Hopkinton, MA, USA). Tumour size (day 8, 21, and 27) and mouse weights (day 29) were also measured and tumour volume was calculated using the following formula: tumour volume (mm^3) = [length x $width^2$]/2 (Faustino-Rocha et al. 2013). As one mouse did not form a tumour, it was excluded from the study.

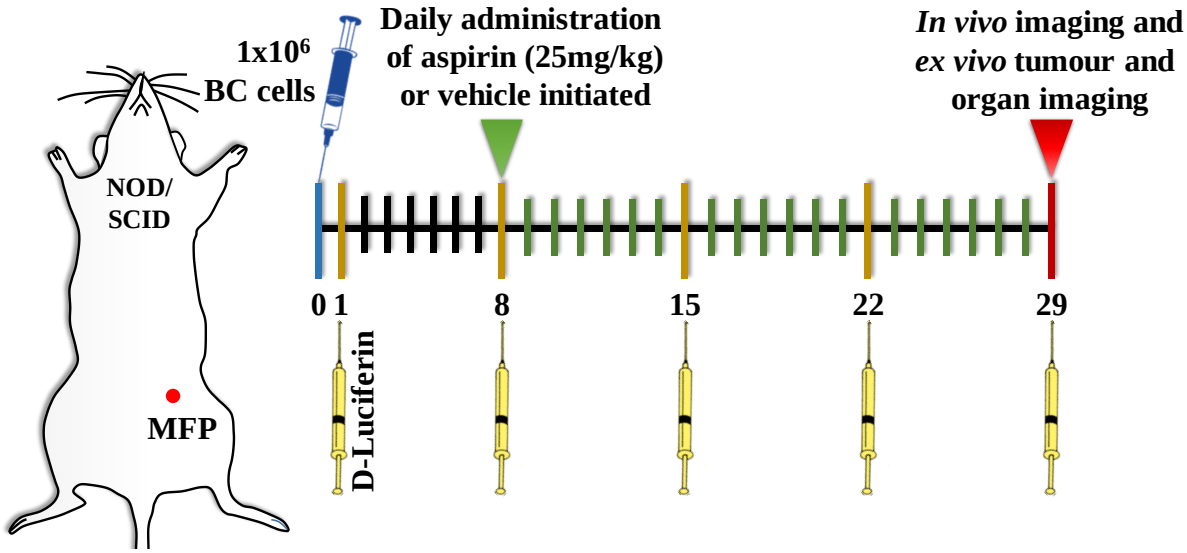


Figure 5-2. Breast cancer model

The 231-LM2 breast cancer cells (BC; $1x10^6$) were injected into the mammary fat pad (MFP) of female NOD/SCID mice. Administration of aspirin (25mg/kg in corn oil, n=11) or vehicle control (corn oil, n=10) via oral gavage was initiated on day 8 and continued to day 29. Mice were injected with D-Luciferin (3mg i.p.) and imaged for luciferase activity on days 1, 8, 15, 22 and 29 post-implantation. At completion of the experiment, tumours and some organs were harvested and re-imaged *ex vivo*.

5.2.1.2 *Aspirin treatment*

Mice were divided into two groups (n=11 mice per group) and received daily treatment of either aspirin (25mg/kg in corn oil) or the vehicle alone (corn oil) via oral gavage. This dose of aspirin in mice was chosen since (a) it corresponds to the average low-dose aspirin in humans (100-150mg dose) using the body surface area (BSA) normalization method (Reagan-Shaw, Nihal & Ahmad 2008), and (b) similar or lower doses are known to inhibit platelet function in mice (Carlson et al. 2013; Guillem-Llobat et al. 2016).

5.2.1.3 *Tumour and organ imaging and harvest*

Mice were humanely killed at the completion of the study and as well as removing the primary tumour, lung, liver, hind limbs and spine were immediately harvested and imaged *ex vivo* for detection of metastases. Organs were imaged within 20 min of luciferase injections, to maximise light signal (Paroo et al. 2004). Samples of primary tumours were weighed then halved with one half being fixed in 10% buffered formalin for 24 hours prior to routine processing and embedding in paraffin wax and the remaining half, as well as the mouse livers, being frozen for gene expression studies (see below).

5.2.2 **Histological and immunohistochemical staining**

Haematoxylin and eosin (H&E) staining was performed on deparaffinised sections using standard protocol (see Chapter 2 section 2.6.4). To stain for platelets, an anti-CD41 polyclonal antibody (Abcam, Cambridge, UK), reactive against both mouse and human platelets, was applied to human blood smears (positive control) at 1/800 dilution and incubated overnight at 4°C. For immunostaining on formalin fixed paraffin embedded (FFPE) tumour sections, the anti-CD41 antibody was used at a 1:1200 dilution. To identify EC-lined vessels and VM, staining for CD31 and PAS was undertaken using previously published methods (Tan et al. 2016). For CD31 staining, sections were incubated with anti-CD31 antibody (Bethyl lab, Montgomery, USA) overnight at 1/250 dilution at 4°C. Immunohistochemistry (IHC) staining protocols are detailed in Chapter 2 section 2.6.5. PAS staining was performed on sections using established protocols. Primary antibody was omitted from negative control sections which were treated with secondary antibody alone. All sections were counterstained with Ehlrich's haematoxylin and subsequently dehydrated, cleared and mounted. Images were captured using an Olympus BX40 microscope fitted with a DP70 digital camera and operated through the Olympus Image Analysis Cell B software program (Olympus, Tokyo, Japan). EC-lined blood vessels (identified as $CD31^{+}/PAS^{+}$) and VM structures (identified as $CD31^{-}/PAS^{+}$) which were defined by the presence of RBCs or WBCs in the lumen. Structures were manually quantified in 4-6 fields of view (FOV) per tumour using ImageJ counter plugin (National Institute of Health, USA), and represented as average structures/FOV.

5.2.3 **TUNEL staining**

The TUNEL assay is a common method used to detect DNA fragmentation due to cellular apoptosis. Apoptotic cells were identified as the enzyme terminal deoxynucleotidyl transferase incorporates fluorescein-dUTP nucleotides to the free 3' ends of fragmented DNA. Tumour sections were stained with the TUNEL assay as described in Chapter 2 (section 2.6.6). Briefly, sections were incubated in TUNEL reaction mixture, fluorescein labelled (Roche Diagnostics, IN, USA), for 60 min at 37°C in the dark then mounted in Vectashield with DAPI (Vector Laboratories). Sections were imaged using the 3DHistech Panoramic Confocal system and analysed with Panoramic Viewer (3DHistech Ltd). Using ImageJ software (National Institute of Health, USA), the total area of the tumour was quantified by drawing around the border of DAPI fluorescence channel, then upper and lower thresholds for the 8-bit image were adjusted to determine total fluorescence in the green channel. The following formula was used to calculate percent positive TUNEL staining: ($TUNEL^+$ area / total tumour area) x 100.

5.2.4 **Gene expression**

The techniques used for gene expression studies are also described in Chapter 2 (section 2.5). Primers for PCR and qPCR were either sourced from the literature (Barrett et al. 2011; Katagiri et al. 2004) or designed using Primer Blast (National Cancer Biotechnology Institute [NCBI]). All primer sequences were validated for species specificity with Primer Blast (NCBI). For conventional PCR, reaction parameters are as stated: 2 min at 98˚C, followed by 34 cycles at 98˚C for 20 seconds, annealing temperature (see Chapter 2, Table 2-2) for 20 seconds, 72˚C for 30 seconds and finally 72˚C for 5 min on a thermocycler (Bio-Rad). Products were electrophoresed at 120V for 45 min on 1.5% agarose gels with QuickLoad 50bp or 100bp DNA ladder (New England

Biolabs, MA, USA). Products were visualised under ultraviolet (UV) light using Uvitec gel documentation system (Uvitec, Cambridge, UK).

For qPCRs, all reactions were completed in a 20µl reaction volume in triplicate. Reaction parameters began with a 2 min hold at 50°C, followed by a 15 min hold at 95°C. Then, a total of 45 cycles were performed as follows; 10 seconds at 95°C, 20 seconds at annealing temperature (Chapter 2, Table 2-2), 30 seconds at 72°C. Data analysis was performed using Rotor-Gene Analysis Software version 6 (Corbett Research). Relative gene expression levels were calculated using the comparative quantitation method available in the Rotor-Gene Software. Gene expression was normalised against the housekeeper genes *Hrpt* and *Cyca* for liver (calibrated using mouse EC RNA), and *GAPDH*, *ACTB*, and *CYCA* for tumours (calibrated using 231-LM2 cells RNA). The suitability of *GAPDH*, *ACTB* and *CYCA* as housekeeper genes was validated using the GeNorm (M value < 1.5) software package (Vandesompele et al. 2002).

5.2.5 **Statistical analysis**

Results were expressed as mean ± standard error of the mean (SEM) from at least 3 experiments. Statistical analysis was performed using GraphPad PRISM software (San Diego, CA, USA). As indicated in the figure legends, data was analysed with Mann Whitney U test, or One-way ANOVA to determine statistical significance with p value <0.05.

5.3 Results

5.3.1 Animal welfare following aspirin treatment

No signs of discomfort were observed in mice in response to the oral gavage and the procedure did not appear to affect food intake. Similarly, no signs of discomfort (hunched posture, rough hair coat, or decreased appetite) were observed in mice receiving either the control or aspirin treatment. As a measure of animal health and well-being throughout the course of the study, mouse weights were taken every two or three days and mean weights were compared between control and aspirin-treated groups. As shown in Figure 5-3, the weight of the mice steadily increased over time, with no significant difference between both groups.

5.3.2 *In vivo* tumour growth of 231-LM2 breast cancer cells

As shown in Figure 5-4 (**A**), bioluminescence heat maps of the mice on days 1, 15 and 29 confirmed that all mice contained 231-LM2 tumours in the MFP and that they continued to grow over time. Quantification of the bioluminescence signal revealed no statistical difference in tumour burden between the control and aspirin-treated mice on days 1 or 8 post cancer cell injection (Figure 5-4, **B**). In contrast, on days 15 and 22 post implantation, a trend was observed wherein aspirin-treated mice exhibited lower tumour burden when compared to the control mice, although this was not statistically significant (Figure 5-4, **B**). By day 29 post implantation, aspirin-treated mice exhibited a significant reduction in tumour burden when compared to the control mice ($P< 0.05$). These bioluminescence imaging results suggest that aspirin administration reduces the number of 231-LM2 breast cancer cells and hence tumour burden.

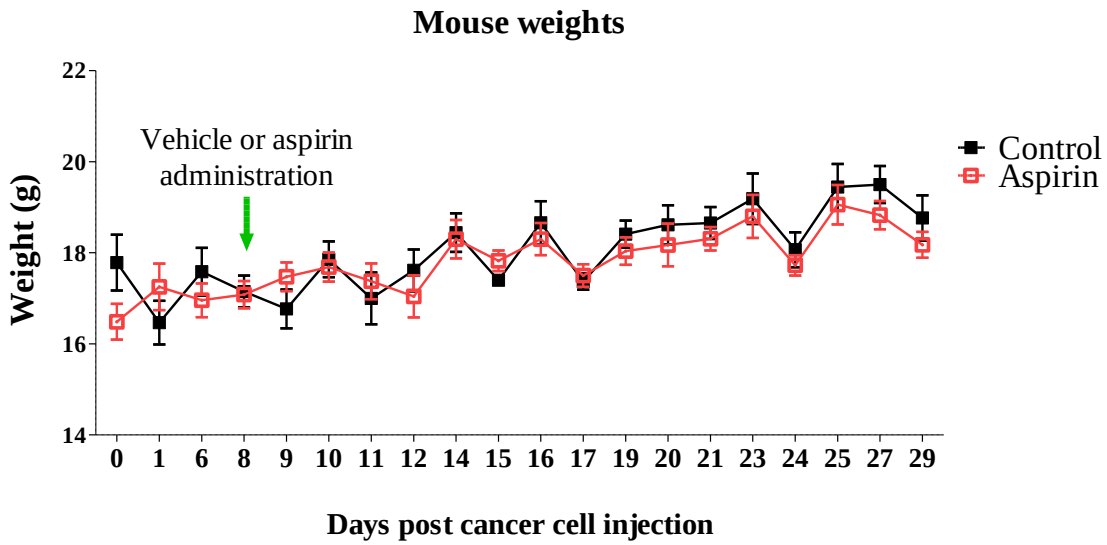


Figure 5-3: Mice weights

Vehicle control (n=10) and aspirin-treated (n=11) mice harbouring 231-LM2 breast cancer xenografts were weighed every two or three days and recorded over time. Results are shown as mean ± SEM.

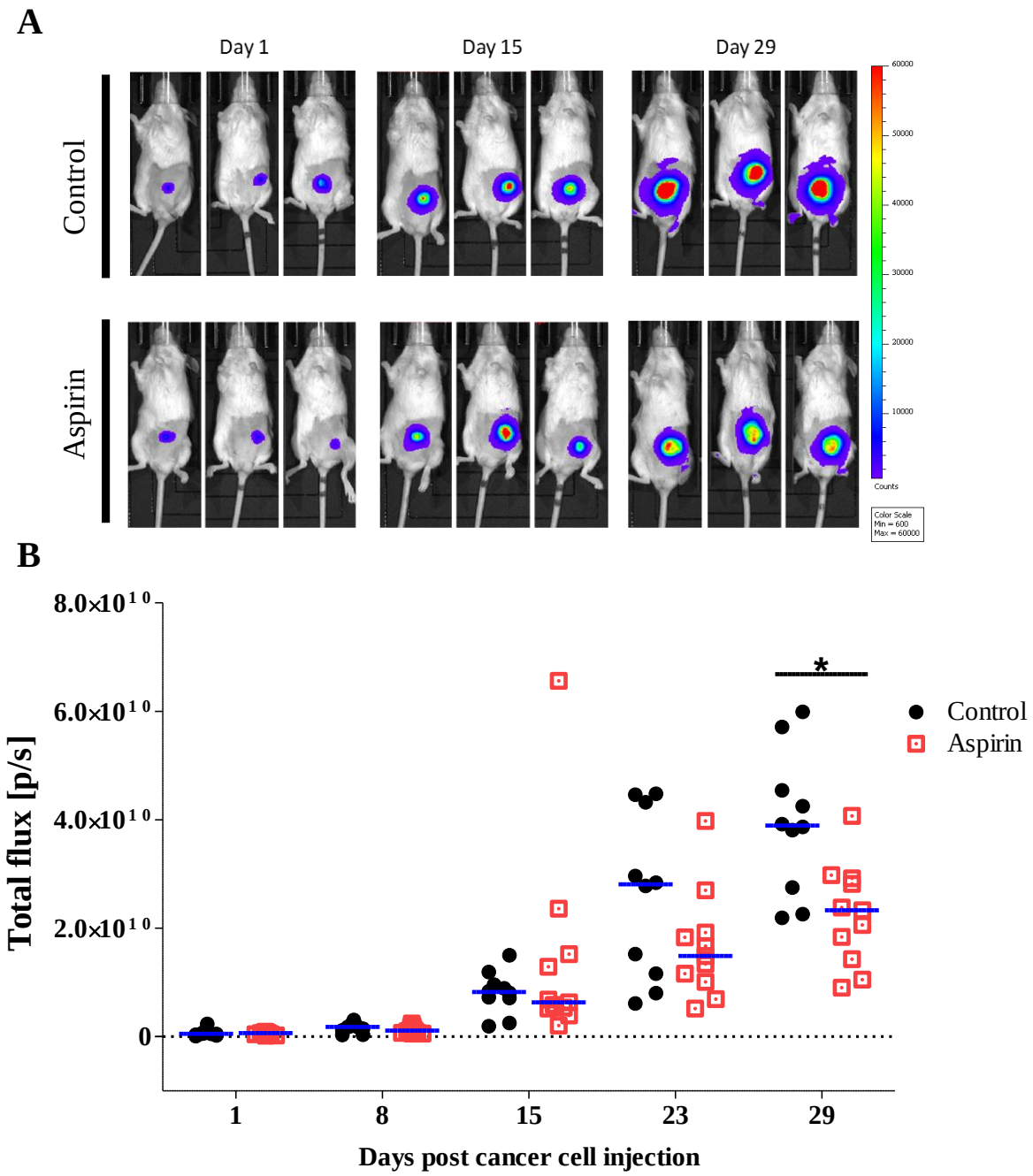


Figure 5-4: Aspirin-treatment reduces *in vivo* tumour burden of 231-LM2 breast cancer cells

(**A**) Representative *in vivo* bioluminescence images of three control (vehicle) and three aspirin-treated mice (25mg/kg) taken on day 1, 15, and 29. Bioluminescent images are shown as a heat maps with blue representing low intensity; red representing high intensity. (**B**) Corresponding quantification of bioluminescence in control (n=10) and aspirin-treated mice (n=11) is expressed as the total flux (photons/second) at each week post 231-LM2 cancer cell injection. Lines in graph represent the median value, **P*<0.05, Mann-Whitney U test.

5.3.3 Tumour volume of 231-LM2 breast cancer tumours in aspirin-treated mice

Tumour volume in the mice, as determined by calliper measurements, reached a maximum size of 250mm^3 (Figure 5-5). Notably, and in contrast to the BLI results, no significant differences were detected in tumour volume between aspirin-treated and control mice at any of the measurement points (day 8, 21, 27). Thus, despite an apparent decrease in tumour cell number (as detected by BLI), aspirin administration did not affect the volume of 231-LM2 tumours in mice over the 27-day measurement period.

5.3.4 Final tumour burden and final tumour weight of 231-LM2 breast cancer tumours in aspirin-treated mice

As shown in Figure 5-6 (**A)**, and similar to whole-body imaging data above, heat maps revealed lower bioluminescence readings in excised tumours from aspirin-treated mice on the final assessment day (day 29). Statistical analysis of total flux also revealed that tumours from mice in the aspirin-treated group exhibited a significant reduction in tumour burden compared with tumours from control mice (Figure 5-6; **B**). However, no significant difference was detected in the weight of tumours excised from control and the aspirin-treated mice (Figure 5-6; **C**). Taken together, these results suggest that although the overall tumour size does not change when the platelets are inactivated, the amount of viable cancerous content in the tumour is reduced.

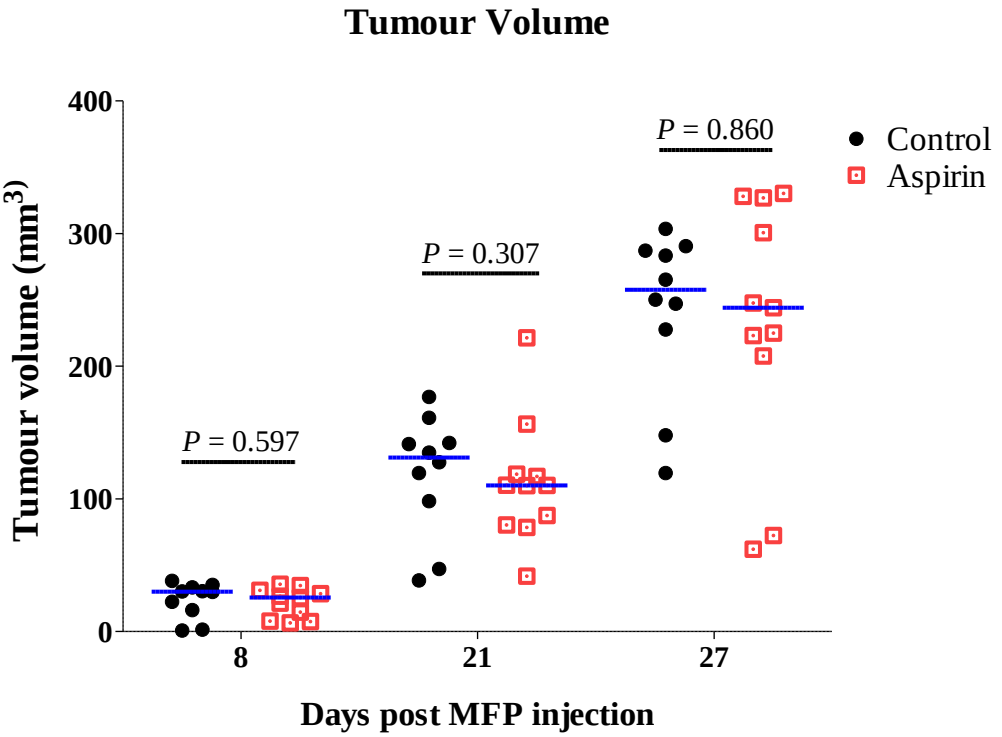


Figure 5-5: Aspirin treatment does not affect volume of 231-LM2 breast cancer over 28 days *in vivo*

Quantification of tumour volume (mm^3) in control (vehicle, n=10) and aspirin-treated mice (25mg/kg, n=11). Tumour volume was measured on days 8, 21, and 27 post 231-LM2 cancer cell injection. Line represents the median. Mann-Whitney U-test.

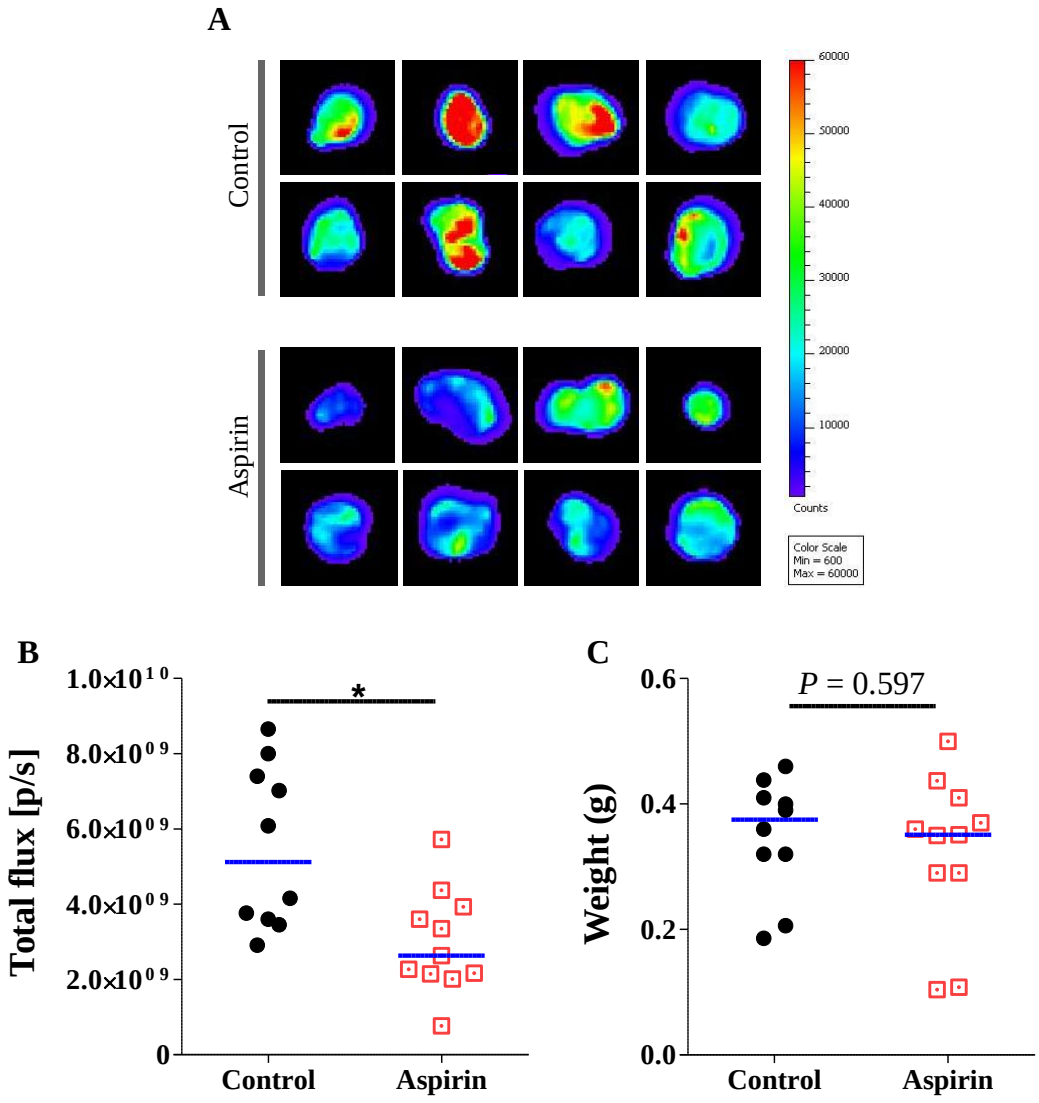


Figure 5-6: Aspirin treatment in mice bearing 231-LM2 breast cancer reduces final tumour bioluminescence signal but not final tumour weight

(**A**) Representative bioluminescence images of excised tumours are shown as a heat maps with blue representing low intensity and red representing high intensity. (**B**) Corresponding quantification of *ex-vivo* bioluminescence expressed as the total flux (photons/second). (**C**) Weights of tumours harvested from control and aspirin-treated mice. Lines in graph represent the median values, *$P < 0.05$*, Mann-Whitney U test.

5.3.5 Histology and apoptosis in 231-LM2 breast cancer cells in aspirin-treated mice

To discern why tumour volume was not significantly reduced in treated mice since BLI showed significantly reduced tumour cell number, histology and assessment of apoptosis were next performed. No discernible differences in cellular composition (leukocytes or stromal cells) or connective tissue were seen in H&E stained sections of tumour from control and aspirin-treated mice (Figure 5-7). However, micro-necrotic regions were more evident in tumours from aspirin-treated mice (Figure 5-7; **C-D**) when compared to tumours from control mice (Figure 5-7; **A-B**).

The TUNEL assay was performed on five tumours from each group to highlight regions of apoptosis. As shown in Figure 5-8 (**A**), all tumours displayed some level of apoptosis. No significant difference in the percentage area of TUNEL positive cells over total tumour area was detected in tumours from aspirin-treated mice in comparison to those from control mice (Figure 5-8; **B**). However, a clear trend towards more apoptosis was evident in the aspirin-treated mice compared to control mice.

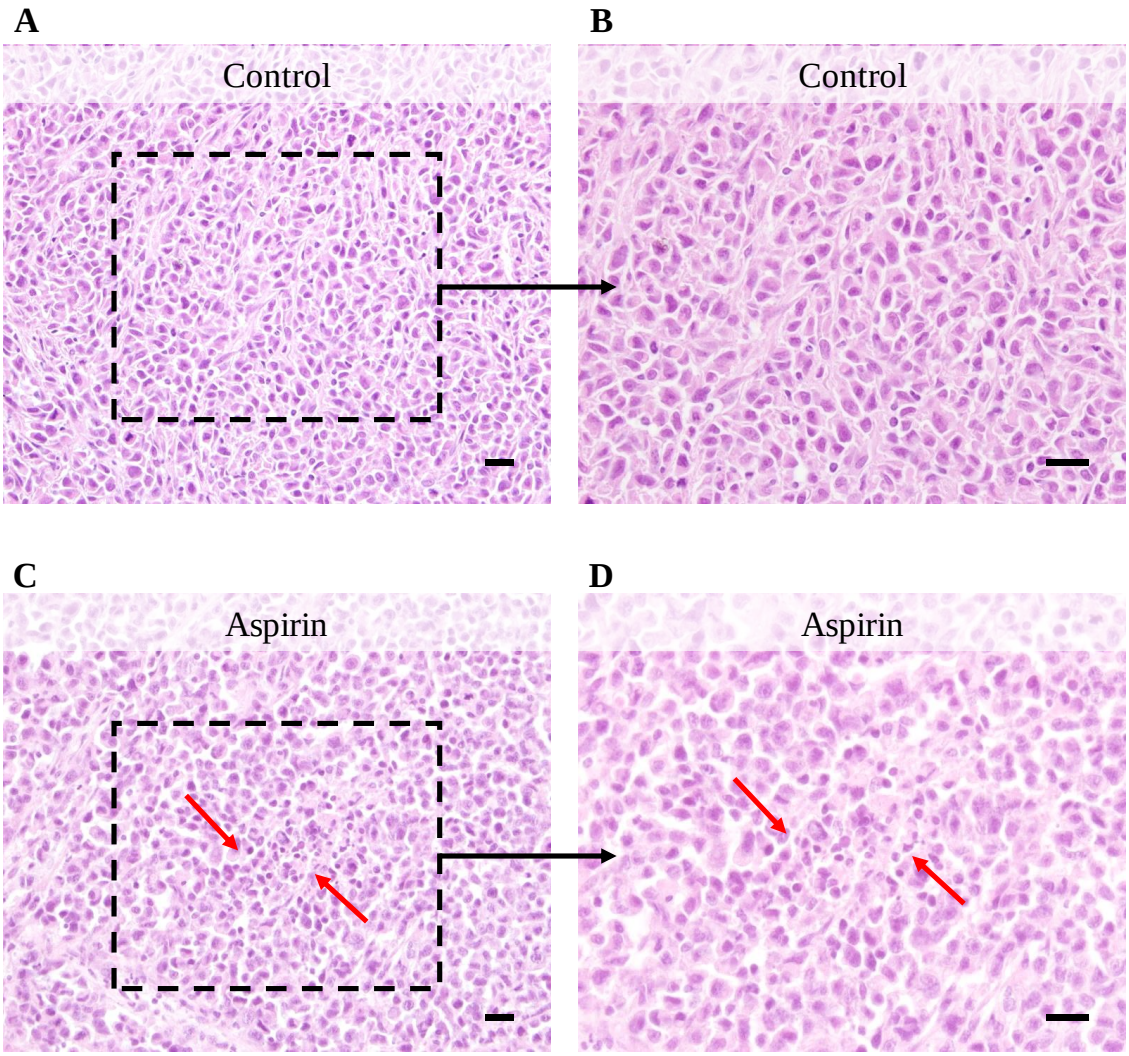


Figure 5-7. Micronecrotic regions are more evident in aspirin-treated mice in the 231-LM2 xenograft breast cancer model.

(**A, B**) Representative images of H&E stained sections of 231-LM2 xenograft tumours from mice treated with vehicle (control) or (**C, D**) aspirin (25mg/kg). Inset in (**A**) is shown in (**B**). Inset in (**C**) is shown in (**D**), demonstrating a micronecrotic region. Micronecrotic region is indicated with red arrows. Scale bars is 20µm, original magnification 400x (**A, C**) and 600x (**B, D**).

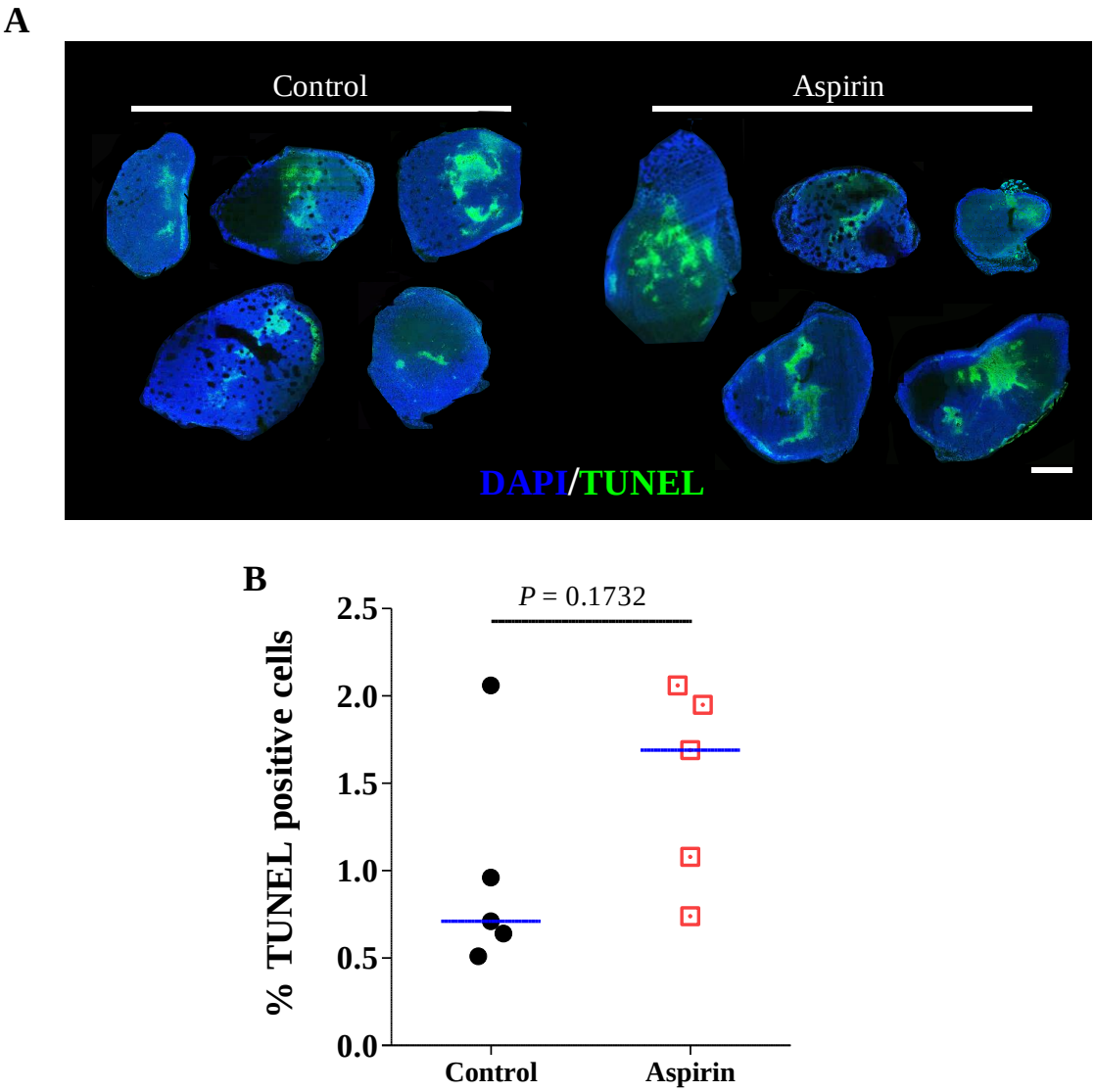


Figure 5-8: Aspirin treatment does not affect apoptosis of 231-LM2 breast cancer cells *in vivo*

231-LM2 xenograft tumours from control or aspirin-treated mice (n=5/group) were assessed with TUNEL assay. (**A**) TUNEL staining of tumour sections from control or aspirin-treated mice. DAPI (blue) fluorescence detects nuclei and FITC (green) fluorescence indicates DNA fragmentation in cells. (**B**) TUNEL positivity quantified (using ImageJ software) as a percentage of total tumour area. Lines in graph represent the medians. Mann-Whitney U test. Scale bar is 2mm, original magnification 20x.

5.3.6 The effect of aspirin on secondary metastases in mice with 231-LM2 breast cancer xenografts

Ex vivo BLI was also performed on the most common sites to which breast cancer metastasises, i.e. lungs, liver, bone and spine. This second round of BLI was captured within 20 min of luciferase injections for the whole-body imaging of the mice and as soon as the organs/tissues were harvested. As demonstrated in Figure 5-9, although more widespread metastases were observed in the lungs harvested from some of the control mice, no significant difference in metastatic load in lung, liver, bone or spine between the control and aspirin-treated mice was found. These data suggest that this dose of aspirin does not affect the metastatic potential of 231-LM2 cells to distant organs.

5.3.7 Detection of platelets in 231-LM2 breast cancer tumours

To investigate whether aspirin treatment had an effect on platelet access to the tumour microenvironment, IHC analysis was performed using a polyclonal antibody against integrin GPIIb (CD41, subunit of GPIIb/IIIa). Initially, the specificity of the anti-CD41 antibody was verified using human blood smears (Figure 5-10, **A-B**) and FFPE sections of human spleen (Figure 5-10, **C-D**). Immunostained tumour sections revealed platelets distributed in intercellular spaces and around the 231-LM2 tumour cells of both the control and aspirin-treated mice (Figure 5-10, E--**H**). The frequency of platelets was similar between the two groups.

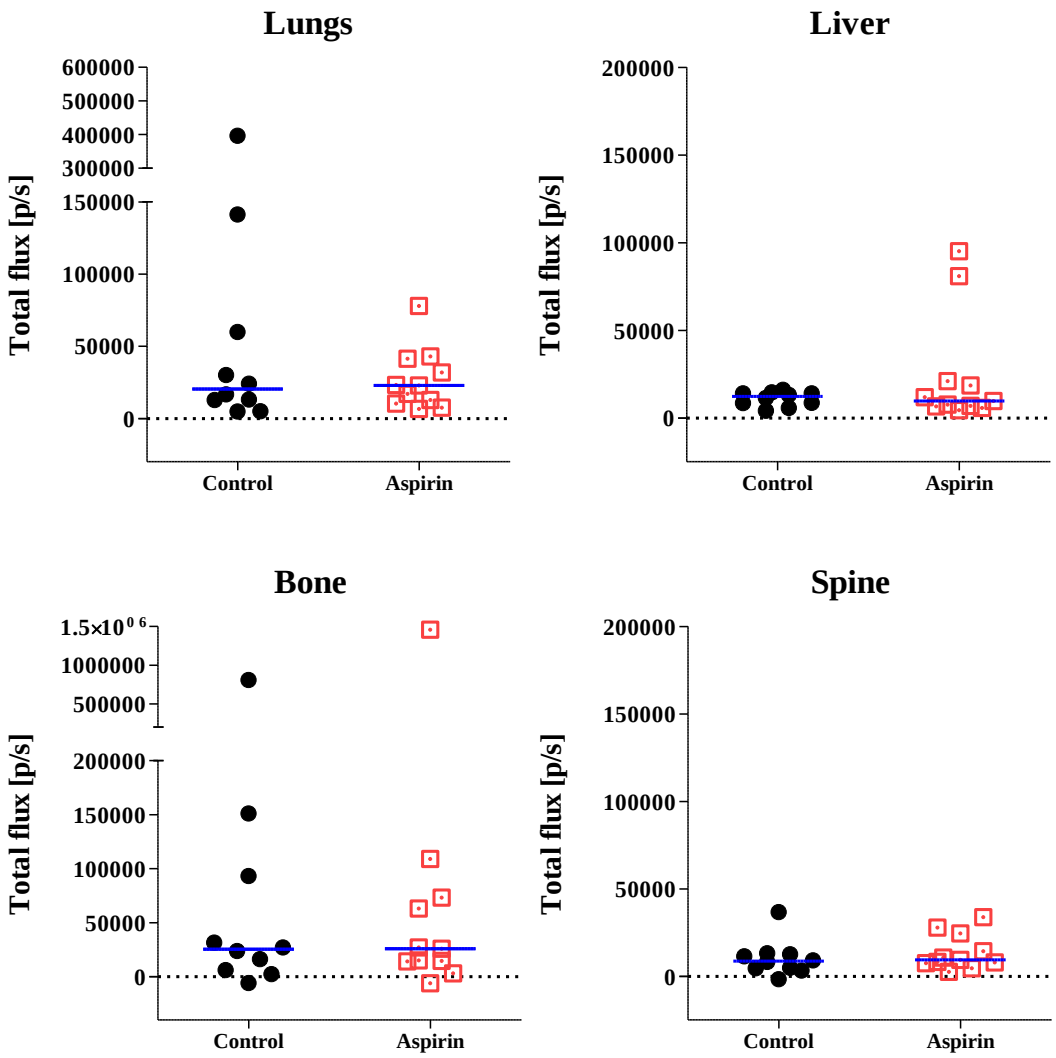


Figure 5-9: Aspirin treatment does not significantly alter metastatic events in mice with 231-LM2 breast cancer

On day 29 post MFP injection of cancer cells, the lungs, liver, long bones of hind legs and spine were harvested from control and aspirin-treated mice (n=10,11/group) and bioluminescence was imaged. Metastatic events were quantified by measuring total flux (photons/second). Lines in graph represent the median values. Mann-Whitney U test.

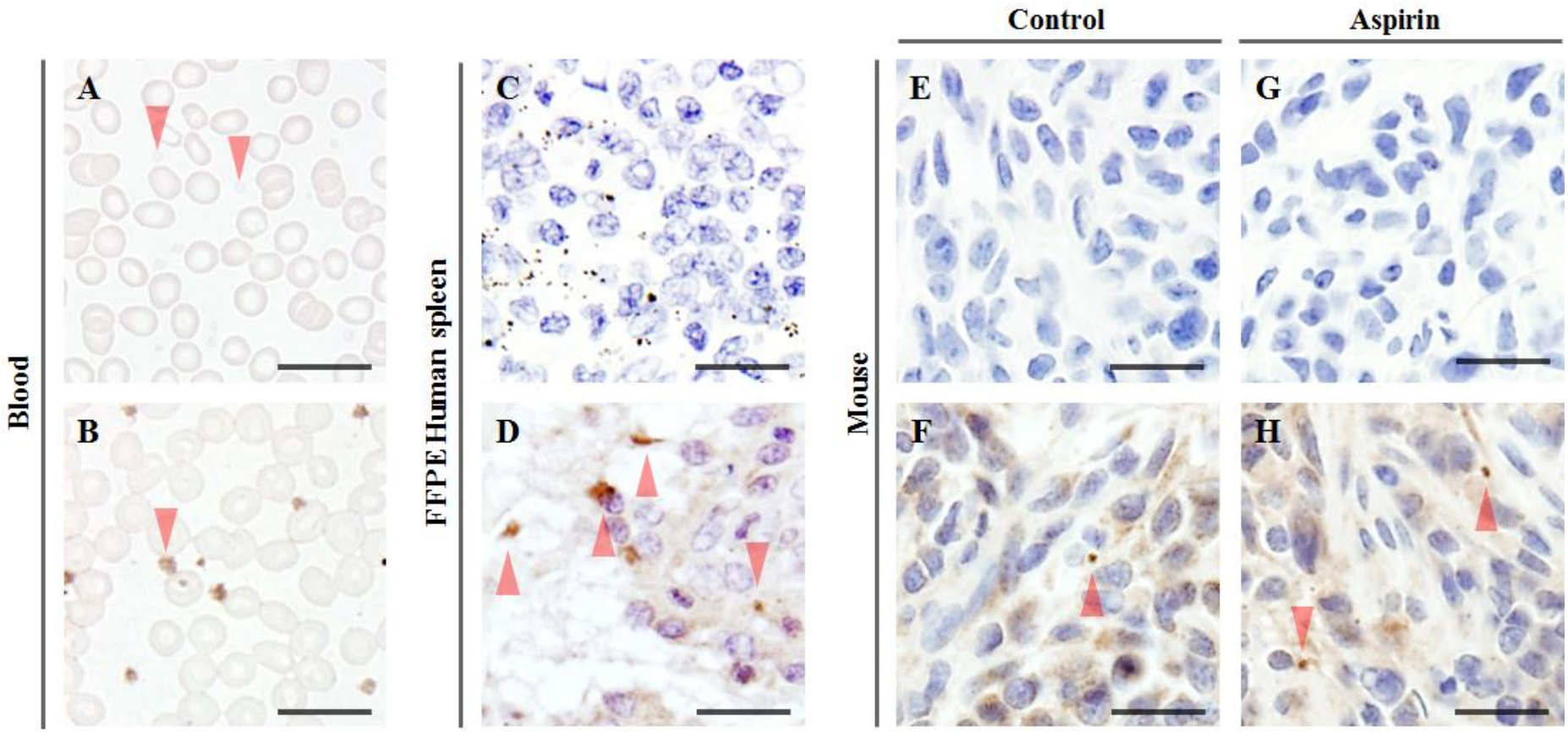


Figure 5-10. Platelets are detected in the tumour microenvironment of 231-LM2 breast cancer in mice with normal or a platelet dysfunction

(**A**) Negative control of blood smear and (**B**) anti-CD41 IHC staining of human blood smear. Platelets are identified by positive brown DAB staining. (**C**) Negative control of human spleen and (**D**) anti-CD41 IHC staining of human spleen. (**E**) Negative control of 231-LM2 breast cancer tumour and (**F**) anti-CD41 IHC staining of tumour from control mouse. (**G**) Anti-CD41 IHC of 231-LM2 breast cancer tumour and (**F**) anti-CD41 IHC tumour from aspirin treated mouse. Red arrow heads indicate platelets. Scale bar is 20µm, 600x original magnification. Images are representative of one stained section from each 231-LM2 breast cancer tumour.

5.3.8 Expression of *Cox-1* and *Cox-2* following aspirin-treatment in mice

Cox-1 and *Cox-2* gene expression were quantified in mice liver samples to assess platelet function in the presence of aspirin, as oral administration has been reported to downregulate *Cox-1* expression in the liver of rats (Fidaleo, Berardi & Sartori 2008). Shown in Figure 5-10 are representative curves of SYBR-green normalised fluorescence for *Cox-1* (**A**) and *Cox-2* (**B**). The average cycle threshold (Ct value) for *Cox-1* expression was cycle 24 for both groups of mice. The average Ct value for *Cox-2* expression was cycles 30 and 32 in control and aspirin-treated mice, respectively. Quantitation revealed no differences in expression of *Cox-1* and *Cox-2* mRNA in the livers of either control or aspirin-treated mice (Figure 5-11, **C- D**). The qPCR studies suggest that low-dose aspirin does not alter the quantity of the COX enzyme at the gene level in mice.

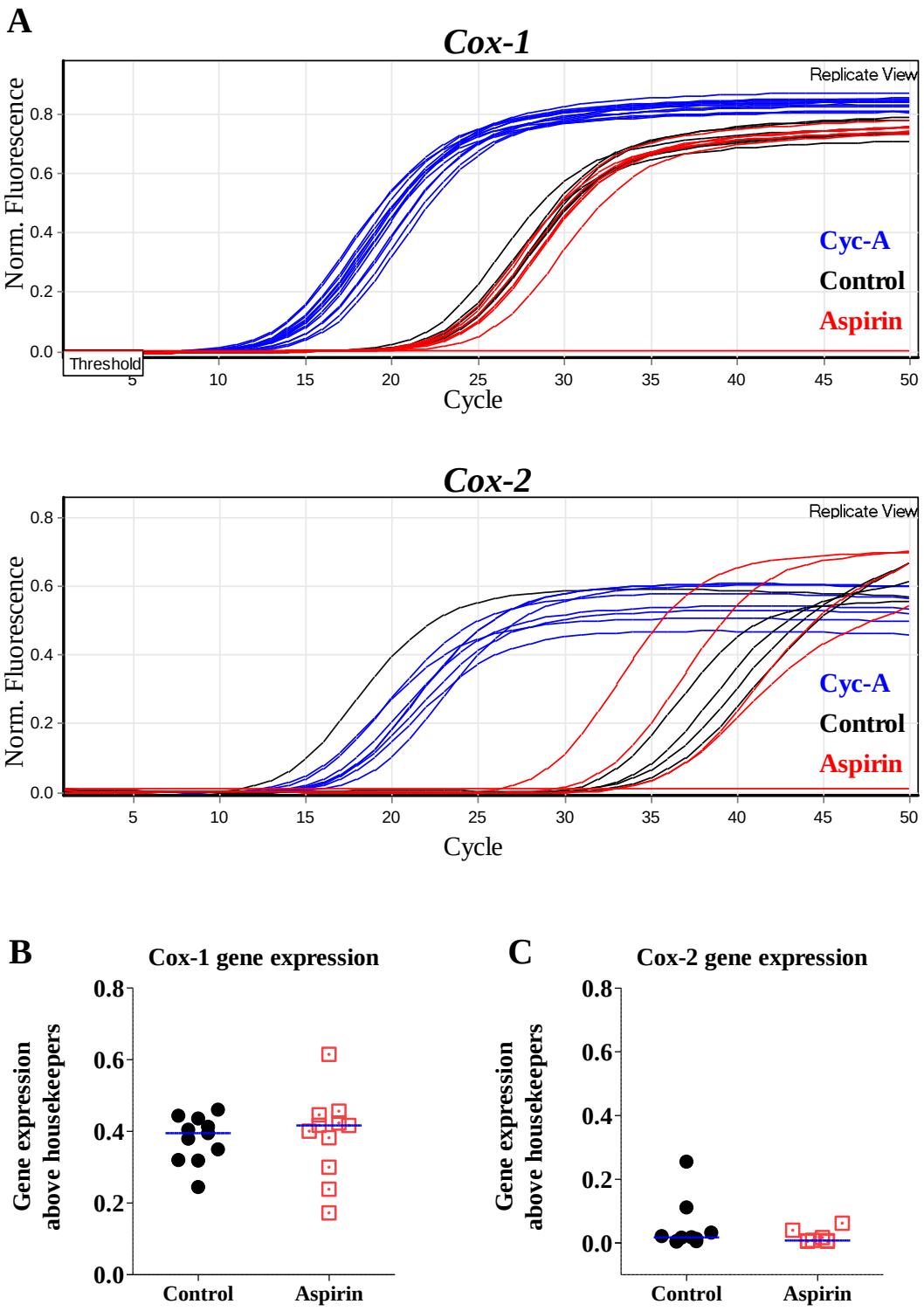


Figure 5-11. *Cox-1* and *Cox-2* gene expression in aspirin-treated mice

(**A**) Representative curves of SYBR-green normalised fluorescence. (**B-C**) Gene expression for *Cox1* and *Cox2* was determined in liver from control and aspirin-treated mice by qPCR. Data are gene expression above housekeepers (*Hrpt* and *CycA*). Lines in graph represent the median. Groups showed no significant difference by Mann-Whitney U test.

5.3.9 The effect of aspirin treatment on EC-lined blood vessels and VM structures in 231-LM2 breast cancer xenograft tumours.

To assess the impact of aspirin treatment on angiogenesis and VM in breast cancer progression, IHC staining was performed on the 231-LM2 tumours harvested from control and aspirin-treated mice using anti-CD31/PAS staining. As shown in Figure 5-12 (**A**), EC-lined blood vessels (angiogenic) were identified by positive immunostaining for CD31 on the luminal aspect and positive PAS staining, while VM structures were identified as PAS positive but CD31 negative.

Quantitation of angiogenic and VM structures revealed no significant difference between the tumours from control and aspirin-treated mice respectively (Figure 5-12, **B**). However, in both control and aspirin-treated mice, ~64% of the vascular structures counted were EC-lined blood vessels (CD31+/PAS+) while the remaining ~36% were VM structures (Figure 5-12, **C**). These results suggest that aspirin treatment does not affect the number of tumour angiogenic structures or VM structures formed by 231-LM2 breast cancer cells *in vivo*.

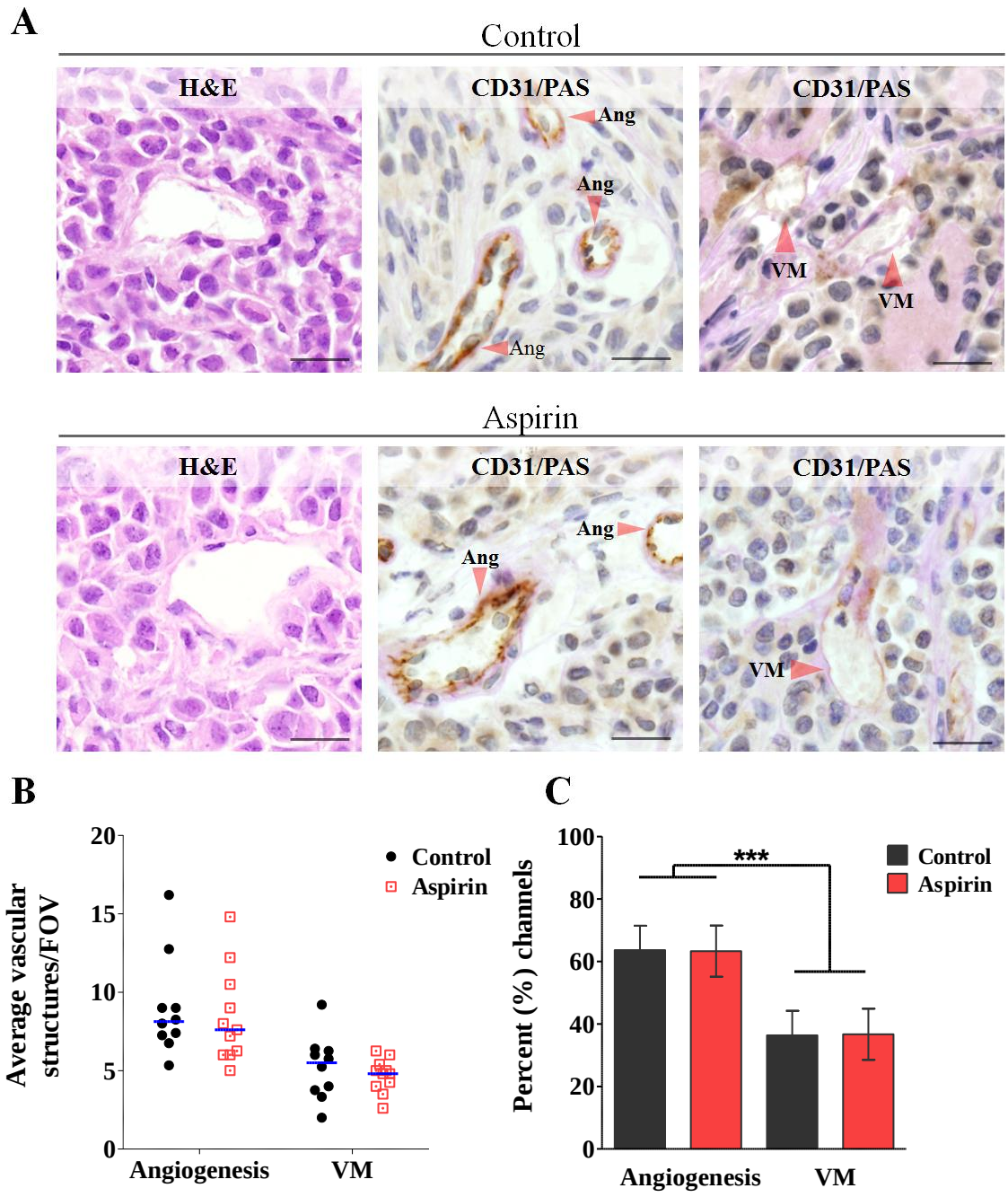


Figure 5-12: The number of VM vessels and EC-lined blood vessels are unaffected in 231-LM2 breast cancer tumours following aspirin treatment

(**A**) 231-LM2 breast cancer tumour tissue sections from control and aspirin treated mice were H&E stained, and IHC stained for CD31 and PAS (n=10-11/group), $CD31^{+}/PAS^{+}$ staining for angiogenic structures and $CD31^{-}/PAS^{+}$ staining for VM structures (see red arrow heads). (**B**) Corresponding quantification of the average angiogenic and VM structures in 5 fields of view (FOV), in tumour sections from control and treatment mice. (**C**) Percent angiogenic vessels and VM structures in control and aspirin-treated mice. Lines in (**B**) represent the medians and in (**C**) represent mean ± SEM, $P<0.0001$, Mann-Whitney U test, 600x magnification, scale bar is 20µm.

5.3.10 Gene expression profile of VM by 231-LM2 breast cancer cells *in vivo* following aspirin-treatment

Lastly, to assess whether aspirin treatment affected the VM gene expression profile, a selected group of genes known to be commonly upregulated and associated with VM occurrence was investigated (Hendrix et al. 2003a; Maniotis et al. 1999). Primers were specifically designed to detect and amplify human genes via qPCR, with no predicted binding to mouse genes.

The expression of human *CDH5* (VE-cadherin), *EPHA2* and *KDR* (VEGFR2) were included to assess whether aspirin affected regulation of the vascular pathway. Expression of *LAMC2*, which is a basement membrane protein upregulated in VM and associated with cell migration and invasion, as well as a collection of matrix-metalloproteases (MMPs) including *MMP1*, *MMP2*, *MMP9*, and *MMP14*, which are involved in remodelling of the extracellular matrix during VM formation, were also investigated. As shown in Figure 5-13, primers yielded products at the expected sizes for *EPHA2* (~100bp), *LAMC2* (~100bp), *MMP1* (~100bp), *MMP2* (~300bp), *MMP9* (~200bp) and *MMP14* (~250bp). Primers specific for *KDR* and *CDH5* were not tested as these were previously validated and published by our research group (Barrett et al. 2011).

Gene expression studies with qPCR revealed that *CDH5* was the most readily detected gene in primary 231-LM2 tumours, and that expression did not differ between the control and aspirin-treated groups of mice (Figure 5-14). Similarly, although *EPHA2, KDR, LAMC2, MMP1*, and *MMP14* expression levels were variable, no significant difference between groups was found. In contrast, expression levels of both *MMP2* and *MMP9* were significantly higher in tumours excised from the aspirin-treated mice. These data suggest

that aspirin treatment does alter the expression of select genes in 231-LM2 breast cancer cells *in vivo* (MMP2 and MMP9) which are part of the VM gene signature profile.

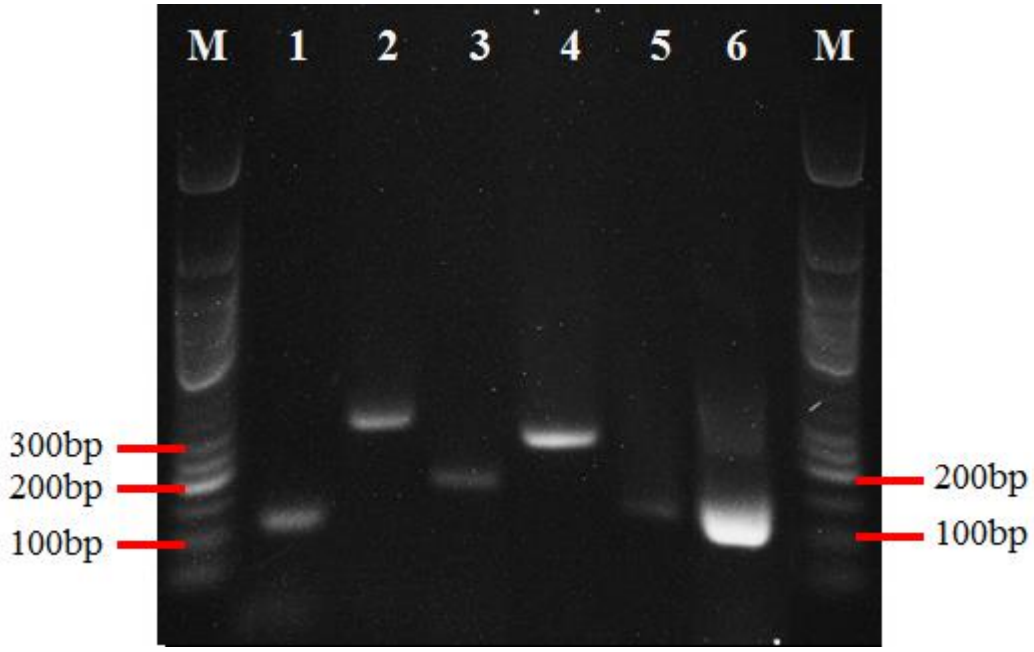


Figure 5-13. Primer specificity with 231-LM2 cancer cell line.

Specificity of primers for genes associated with a VM profile was tested using 231-LM2 human breast cancer cDNA. PCR products were run on a 1.5% TAE-agarose gel. Lanes: (**1**) *MMP1* gene expression, band detected at ~100bp, (**2**) *MMP2* gene expression, band detected at ~300bp, (**3**) *MMP9* gene expression, band detected at ~200bp, (**4**) *MMP14* gene expression, band detected at ~250bp, (**5**) *LAMC2* gene expression, band detected at ~100bp, (**6**) *EPHA2* gene expression, band detected at ~100bp, (**M**) Represents QuickLoad 50bp DNA ladder.

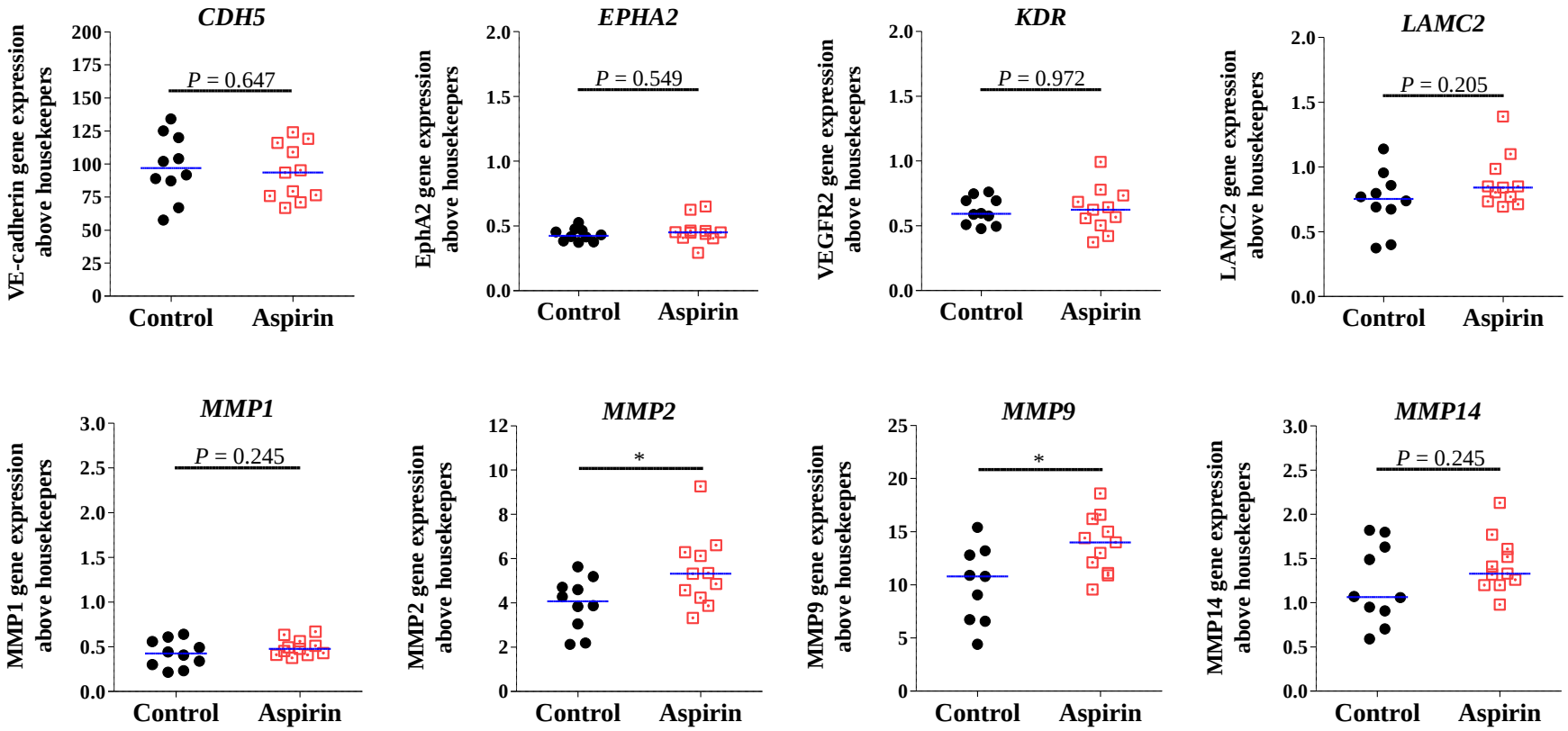


Figure 5-14: Platelet dysfunction does not affect the gene expression profile of VM by 231-LM2 breast cancer cells *in vivo*

RT-qPCR was used to determine gene expression in 231-LM2 breast cancer tumours from control and aspirin treated mice (n=10,11/group). Scatter dot plot showing the mRNA gene expression above housekeeping genes (*GAPDH*, *ACTB*, and *CYCA*), of eight VM-associated genes; *CDH5*, *EPHA2*, *KDR*, and *LAMC2*, *MMP1*, *MMP2*, *MMP9*, and *MMP14*. Lines in the graph represent the median values, **P*<0.05, Mann-Whitney U test.

5.4 **Discussion**

Amongst all the breast cancer subtypes, patients with TNBC have the poorest prognosis (Dent et al. 2007; Zhang et al. 2014a). This is largely due to their lack of expression of HR (ER and PR) and HER2 such that chemotherapy is the only treatment option available for these patients. Even then, significantly more patients treated ultimately relapse compared with non-TNBC (Haffty et al. 2006; Liedtke et al. 2008). This has driven the need to find therapeutic targets in TNBC which provide tangible treatment opportunities.

Studies in Chapters 3 and 4 showed that platelets inhibit VM formation by TNBC cells *in vitro*, suggesting that platelets may provide a potential tool against TNBC *in vivo*. In the current Chapter, it was demonstrated that low-dose aspirin, documented to inhibit platelet function in mice (Carlson et al. 2013; Guillem-Llobat et al. 2016), reduced tumour burden in a xenograft model of TNBC possibly by increased neoplastic cell apoptosis in primary tumours. Although *MMP2* and *MMP9* mRNA expression was elevated with aspirin treatment, no differences in tumour volume and weight, the percent of angiogenic and VM structures, and metastases were observed between control and aspirin-treated mice. Evidently, *in vitro* data showed that platelets inhibit VM formation, but no differences in VM were found *in vivo*. The *in vitro* data may not be reflected *in vivo* due to the difference in models i.e. very controlled conditions (*in vitro*) versus a less controlled and more complex system (*in vivo*).

A 25mg/kg aspirin dose in mice was selected for these studies to investigate the specific effect of platelet function. This corresponds to a low-dose aspirin in humans based on the body surface area (BSA) normalization method, which is a more accurate means of extrapolating human-to-mouse dose compared to a simple conversion based on body weight (Reagan-Shaw, Nihal & Ahmad 2008). As stated above, published studies wherein

aspirin has been administered to inhibit platelet function in mice have not measured platelet function (Kuznetsov et al. 2012), in fact, very little is done to confirm platelet inactivation.

With commitments to prove that aspirin-treated mice had inactivated platelets, a number of approaches were undertaken. Firstly, blood clotting time, although a crude test for platelet function, was assessed based on other studies showing oral doses of aspirin as low as 18mg/kg increased bleeding time in mice (Itzhaki et al. 2013). However, inconsistencies in bleeding time in both groups of mice (data not shown) deemed this protocol to be unreliable. As an alternative approach, *Cox-1* and *Cox-2* mRNA levels in liver samples were used as a marker of aspirin effect on platelet function, as aspirin is reported to downregulate *Cox-1* in the liver (Fidaleo, Berardi & Sartori 2008). *Cox-2* mRNA showed no difference between groups, which was as expected as the protein is only significantly inhibited with high-dose aspirin (>160mg/day in humans, >32mg/kg in mice) (FitzGerald et al. 1983; Patrono et al. 2008). In contrast to expectations, *Cox-1* expression also did not differ between the groups. This suggests that a 25mg/kg dose is too low to have a measurable effect on *Cox-1* gene expression levels, since the study mentioned above observed differences using a significantly higher dose of aspirin (~1000mg/kg) (Fidaleo, Berardi & Sartori 2008).

While a higher aspirin dose may inhibit *Cox-1* gene expression, a 25mg/kg dose was chosen for the following reasons; firstly, it corresponds to low-dose aspirin in humans (100-150mg) which has specific platelet-inhibitory function and no off-target effects (e.g. analgesic and anti-inflammatory); and secondly, elevated doses (80-150mg/kg) lead to a corresponding increase in liver toxicity in mice (Patrono et al. 2008; Vad et al. 2014). While low-dose aspirin (25mg/kg) did not regulate *Cox-1* and *Cox-2* gene expression, this does not necessarily mean that *Cox-1* is unaffected at the protein level with a 25mg/kg

dose of aspirin. This is based on the finding that oral doses of aspirin as low as 10 mg/kg lead to a significant reduction in TXB_2 (Carlson et al. 2013) which is a stable metabolite of TXA_2 and an indicator of platelet COX-1 activity (Patrignani, Filabozzi & Patrono 1982). Furthermore, an oral dose of 20mg/kg almost completely suppresses TXB_2 (Guillem-Llobat et al. 2016), suggesting that an oral dose of 25mg/kg may well have had an inhibitory effect on platelet function in mice throughout the duration of the current study. Measuring plasma TXB_2 levels may therefore be a better method for confirming the platelet inhibitory effects of aspirin in future studies.

Large prospective observational trials have shown that aspirin use improves survival in patients with breast cancer (Holmes et al. 2010) and, in stage II and III TNBC patients, improved rate of distant metastases and DFS (Shiao et al. 2017) although the dose and administration regimen was not mentioned in either report. In the current study, mice treated with low-dose aspirin (25mg/kg) (Carlson et al. 2013; Guillem-Llobat et al. 2016), had reduced tumour burden (as measured by BLI) at completion of the experiment (day 29) when compared to control mice. Notably, caliper measurements and final tumour weights failed to support this observation of reduced tumour burden, with no significant differences observed between the aspirin-treated and control mice. This suggests that although the size of the tumour did not differ between groups, the cancer content within the tumour mass was different. Indeed, monitoring subcutaneous tumours by caliper measurements does not consider the occurrence of necrosis, ulceration, or apoptosis within the tumour, making BLI a more sensitive tool to monitor the presence of viable cancerous cells over time (Craft et al. 2005).

In agreement with this, investigation of the excised tumours revealed more micronecrotic regions in the tumours from aspirin-treated mice, and TUNEL staining showed increasing levels of apoptosis/necrosis in the tumours from the aspirin-treated mice when compared to the controls, although this was not statistically significant. Only five tumours from each group were assayed, which limits the opportunity for a significant difference to become evident. In ovarian cancer cells, Egan and colleagues demonstrated that platelets and their released contents can upregulate expression of anti-apoptotic and anti-autophagy genes (Egan et al. 2011). Therefore, the studies in this chapter suggest that the induction of apoptosis in the TNBC model following low-dose aspirin treatment may signify an inhibition of such protective properties mediated by platelets towards the survival of tumour cells.

It is also important to consider that aspirin may directly act on cancer cells leading to apoptosis as shown in mouse models of colon cancer using a 25mg/kg dose (Reuter, Zhang & Miller 2002), and TNBC using a dose of 75mg/kg (Maity et al. 2015). In the latter study, this effect was due to activation of caspase 3 (Maity et al. 2015). There is also evidence showing that aspirin can induce apoptosis through other mechanisms which affect mitochondrial activity, including inhibition of proteasome function, release of cytochrome C and closure of the voltage-dependent anion channel-1 (Dikshit et al. 2006; Pique et al. 2000; Tewari et al. 2017; Zimmermann et al. 2000). Therefore, together with the work by others, the findings of this Chapter suggest that low-dose aspirin leads to a reduction in tumour growth, an effect which may occur through indirect (platelet inhibition) or direct (apoptosis induction) actions.

Platelets are known to enter the tumour microenvironment and come into direct contact with ECs and tumour cells in a variety of cancers including breast, melanoma, colorectal, and others (Li et al. 2014a; Manegold et al. 2003; Qi et al. 2015; Stone et al. 2012; Yuan & Liu 2015). Furthermore, studies have also shown that aspirin treatment does not alter the number of circulating platelets in mice (Kuznetsov et al. 2012). This suggests that, even in the presence of aspirin, platelets may enter the tumour microenvironment. Indeed, the IHC studies in the current work show that platelets enter the microenvironment of TNBC and associate closely with cancer cells in both aspirin-treated and control mice. The frequency of platelets in the microenvironment was similar between the two groups. The IHC studies also showed that low-dose aspirin had no effect on the number of vascular structures in the tumour compared with control mice. This is also reflected in the consistency in overall tumour volume and is also consistent with studies by Dong and colleagues showing that, in a SCID mouse model of angiogenesis, >22% apoptotic cells were required for a significant reduction in microvascular density *in vivo* (Dong et al. 2007).

Vasculogenic mimicry structures anastomose with EC-lined tumour vasculature or obtain fluid from leaky vessels to actively perfuse the tumour (Frenkel et al. 2008; Hendrix et al. 2003b; Ruf et al. 2003; Shirakawa et al. 2002a; Shirakawa et al. 2003). The studies in Chapter 3 demonstrated that platelets are inhibitory towards VM structure formation by TNBC cells *in vitro*. Based on this *in vitro* observation and that low-dose aspirin inhibits platelet function (Patrignani, Filabozzi & Patrono 1982), it was hypothesised that low-dose aspirin would thereby promote VM formation *in vivo*. However, the studies in this Chapter showed that platelet inactivation did not increase the proportion the VM content in the primary tumours. In fact, no difference in total tumour vasculature (angiogenic or VM) was identified. It may be that the approach of manually quantifying VM structures

($CD31^{-}PAS^{+}$) in FFPE sections of primary tumours by IHC does not provide an accurate assessment (Buijs et al. 2004; Hendrix et al. 2001) although at present $CD31^{-}/PAS^{+}$ IHC remains the most applicable method available for assessing VM formation *in vivo* (Yang et al. 2016). Since no differences in VM formation were observed between control mice and mice on aspirin treatment, the *in vitro* (Chapter 3) and *in vivo* studies (Chapter 5) collectively suggest that the role of platelets in VM formation by TNBC may be multifactorial and further studies are needed to elucidate the processes involved.

Tumour cells lining VM channels are in direct contact with circulating blood (Shirakawa et al. 2003), and the presence of VM in a range of malignancies, including TNBC, is associated with metastasis and poor patient survival (Liu et al. 2013b; Zhang et al. 2014a). The most common sites of secondary metastases in breast cancer are lungs (~50% of cases), followed by liver (~26% of cases), and the remaining (~24%) in bone and brain (Pinessi et al. 2015). The TNBC cell line, MDA-MB-231, is known to metastasise to multiple sites including the lungs and liver in the NOD/SCID tail-vein injection model (Li et al. 2014b; Wagenblast et al. 2015). Although differences were not statistically significant, animals treated with low-dose aspirin exhibited fewer lung metastases when compared with control mice. The use of the MFP model may explain the modest effects observed within the time course, thus a better model to investigate metastasis involves resecting the primary tumour thereby allowing sufficient time for disseminated cells from the primary tumour to develop into fully established and easily detectable metastases. Nevertheless the findings are supported by previous *in vivo* studies in which platelet depletion or inhibition with aspirin reduced lung metastases in models of 4T1 (TNBC), B16 melanoma, and HT29 human colon carcinoma (Coupland, Chong & Parish 2012; Guillem-Llobat et al. 2016; Li et al. 2014a; Lonsdorf et al. 2012). Although not significant, there was also an indication that aspirin caused more widespread liver

metastasis, as noted in liver metastases of B16 melanoma in NK depleted mice (Coupland, Chong & Parish 2012). These authors postulated that depletion of platelets allowed cancer cells to pass more easily through the lungs and to the liver due to a reduction in platelet-assisted binding to endothelium. Liver metastases may occur independently of the action of platelets in this *in vivo* model of TNBC since aspirin is also shown to inhibit expression of GPIIb/IIIa and P-selectin (Isenberg et al. 2007), both important in platelet-assisted cancer cell metastasis to the lungs (Kim et al. 1998a; Lonsdorf et al. 2012; Qi et al. 2014; Trikha et al. 2002). Collectively, the data suggest that 231-LM2 metastasis to the lungs is dependent on platelet function whilst liver metastasis is not as it increases when platelet function is compromised. The findings, together with work by others, emphasise the complexity of platelet-dependent organ metastasis and highlight a role for platelets in assisting metastases from the primary tumour to secondary distant sites.

Several genes are known to be upregulated during VM including *CDH5* (VE-cadherin), *EPHA2*, *LAMC2*, *VEGFR2*, and MMPs (*MMP1*, *MMP2*, *MMP9*, and *MMP14*) (Hendrix et al. 2003a; Seftor et al. 2002b; Seftor et al. 2001; Sood et al. 2004; Wang et al. 2012). There were no obvious changes in expression level for *CDH5*, *EPHA2*, and *KDR* upon aspirin treatment. The gene which encodes for laminin 5γ2-chain, *LAMC2*, was slightly elevated in tumours from aspirin-treated mice. In VM, laminin 5γ2-chains are cleaved by MMPs (MMP2 and MMP14) into promigratory γ2' and γ2x fragments which promote migration, invasion and VM formation (Seftor et al. 2002c; Seftor et al. 2001). The expression of *MMP2* was significantly higher in aspirin-treated mice whilst *MMP14* showed a trend towards an increase. In addition, *MMP9* expression was significantly higher in tumours from aspirin-treated mice. Mehner and colleagues showed that knockdown of both MMP2 and MMP9 inhibited the invasive activity of MDA-MB-231 cells *in vitro* (Mehner et al. 2014). Similarly, silencing MMP9 in the MFP xenograft

model (231-LM2) inhibited metastasis to the lungs 11-weeks after cancer cell injection (Mehner et al. 2014). The gene analysis data in this Chapter suggest that low-dose aspirin may induce a more aggressive and invasive phenotype in TNBC 231-LM2 cells via elevation of MMPs.

The data in this Chapter suggest that low-dose aspirin had inhibitory effects on tumour burden, possibly through inducing increased neoplastic cell apoptosis in primary tumours. Whether this reflects an inhibition of the cancer cell-protective properties of platelets or is a result of inhibiting platelet activity is not clear and remains to be determined. The inner region of solid tumours is often hypoxic, and hypoxia-induced apoptosis is commonly found (Vaupel, Mayer & Hockel 2004). Studies have shown however that cancer cells can survive in hypoxic microenvironments through translocating hexokinase II (HK-II) to the voltage-dependent anion channel-1 (VDAC1) in the mitochondria and thus enabling anaerobic metabolism (Pedersen 2008). Aspirin is shown to dissociate HK-II from VDAC1, leading to cell death in cervical (HeLa) and oral cancer (SCC-131) cell lines (Tewari et al. 2017). This suggests that low-dose aspirin may contribute to hypoxia-induced cell death. Additionally, preliminary studies by Lienard and colleagues observed that aspirin can be metabolised into inhibitors of hypoxia-inducible transcription factor (HIF) hydroxylase (Lienard et al. 2008). By blocking degradation of HIF-1α, aspirin inactivates the hypoxic response leading to accumulation of HIF-1α in cells.

Notably, HIF-1α elevates MMP2 and MMP9 expression in a variety of malignancies including TNBC MDA-MB-231 cells, which then promotes cancer spread and survival throughout the body (Choi et al. 2011; Lee et al. 2014; Zong et al. 2016). Several genes are known to be regulated by hypoxia to promote angiogenesis, including *VEGF* (Lindberg et al. 1993; Rebres et al. 2001). In contrast, aspirin has been shown to inhibit VEGF release from platelets (Battinelli, Markens & Italiano 2011; Coppinger et al. 2007).

Since no effect by low-dose aspirin was observed on tumour angiogenesis or VM formation in the TNBC 231-LM2 xenograft model used here, this may suggest that low-dose aspirin (by inhibiting release of contents from platelets) induces only modest levels of hypoxia in the microenvironment, insufficient to affect HIF-1α and thus tumour perfusion.

5.5 Summary

The original hypothesis of this Chapter was that a platelet inhibitory dose of aspirin would promote VM formation by TNBC *in vivo*. While the IHC experiments did not show changes in VM or tumour angiogenesis, the gene expression studies provide an indication that low-dose aspirin induces elevated expression of genes associated with an aggressive and invasive phenotype and this could potentially influence VM formation.

Chapter 6. Final discussion and concluding remarks

For tumours to grow beyond $2mm^3$, they must obtain a blood supply for the provision of oxygen and nutrients (Folkman 1971). Tumours can achieve this through promoting the growth of pre-existing EC-lined vasculature towards and into the tumour. However, this can also occur through other mechanisms including vasculogenic mimicry (VM), whereby cancer cells can produce their own basement membrane and arrange into vascular-like structures (Maniotis et al. 1999). Such structures can anastomose with normal vasculature and supply blood to the growing tumour.

It is now well documented that VM can occur in a variety of cancers, including melanoma, breast, ovarian, colorectal, lung, mesothelioma and others, and is found to associate with poor cancer differentiation, lymph node and distant metastasis, TNM stage, and reduced overall survival in patients (Cao et al. 2013; Yang et al. 2016). Currently there is one targeting compound effective against VM (CVM-1118; TRX 818) in Phase I clinical trials (Hendrix et al. 2016) which are currently ongoing in the US and Taiwan and are yet to report results. With only one therapeutic agent being tested, there remains a desperate need to find new therapeutic strategies to target VM. The use of angiogenic inhibitors has had no effect on VM *in vitro* (van der Schaft et al. 2004), and in fact has reported a more aggressive progression of cancer xenograft models *in vivo* in which VM has been noted (Ebos et al. 2009; Paez-Ribes et al. 2009; Sun et al. 2017; Xu et al. 2012; Zhang et al. 2014a). Taken together, this identifies a current unmet need to target not only angiogenesis but also VM.

Platelets play a crucial role in maintaining haemostasis and contain an abundance of different molecules which are involved in numerous physiological and pathological processes, including tumour angiogenesis (Brill, Elinav & Varon 2004; Coppinger et al. 2004; Sabrkhany, Griffioen & Oude Egbrink 2011). Although platelets have the capacity to release both pro and anti-angiogenic regulators (Chatterjee et al. 2011; Coppinger et

al. 2004; Italiano et al. 2008), several studies have focussed on their pro-angiogenic role, demonstrating that they assist tumour growth while supporting EC-lined tumour angiogenic vessels and promoting metastasis (Ho-Tin-Noe et al. 2008; Nieswandt et al. 1999). This is in line with evidence that elevated platelet numbers are commonly found in cancer patients and correlate with poor prognosis (Ikeda et al. 2002; Pedersen & Milman 1996; Shimada et al. 2004; Sirott et al. 1993). However, studies have also demonstrated that platelets, possibly via their TSP-1 content, suppress tumour growth by inhibiting angiogenesis in murine models of lung cancer and melanoma (Zaslavsky et al. 2010).

Despite many studies investigating the role of platelets in cancer, the present study is the first to investigate whether platelets regulate VM by cancer cells. The overall aim of this study was to investigate this phenomenon thus contributing to current findings and the development of new therapeutic strategies to treat cancer. More specifically, these studies sought to investigate the effect of platelets on VM both *in vitro* and *in vivo*, as well as elucidating the mechanisms involved. The following sections provide a summary of the major findings of this research, the implications of these findings, the limitations and recommendations for future research, and concluding remarks.

6.1 Summary of the major findings

In Chapter 3, the effect of platelets on VM was assessed using the tube formation assay, (Geltrex), which is the gold standard for investigating EC angiogenesis and VM *in vitro* (Francescone, Faibish & Shao 2011). A series of centrifugation and washing steps were utilised to purify human platelets collected from healthy donors and the quality of platelets was validated using flow cytometry wherein the platelets expressed high levels of CD42b (a platelet-specific marker) and low levels of P-selectin (a marker of platelet activation). Co-culturing platelets with cancer cells in the Geltrex assay revealed an anti-VM effect mediated by platelets for MDA-MB-231 and HS-578T breast cancer cell lines and the C32 melanoma cell line. Notably, platelets did not inhibit VM by the CHL-1 melanoma cell line. This suggests a selective effect of platelets on VM in cancer. The most profound effect was observed with C32 melanoma cells wherein the presence of platelets in the ratio of 1:20 (cancer cells: platelets) completely inhibited VM formation, an effect that was not observed using this same ratio with MDA-MB-231 or HS-578T breast cancer cell lines. Further investigation revealed that this inhibitory effect was mediated through direct interaction of platelets with the cancer cells as replacing platelets with inert platelet-sized (2µm) beads did not inhibit VM formation.

Chapter 4 then went on to describe a mechanism underpinning the effect of platelets on VM inhibition. Time-lapse imaging revealed three stages of the VM process wherein cancer cells in the tube formation assay start as single cells which begin to change shape and make contact with neighbouring cells (Stage 1), before elongating and interconnecting (Stage 2), and finally forming multi-cellular inter-connecting vascular-like structures (Stage 3). The stages observed in VM formation are similar to those observed in the late stage of EC angiogenesis *in vitro* using the same or similar assay by others (Aranda & Owen 2009; Bahramsoltani et al. 2010). Time-lapse imaging revealed

that platelets inhibited Stage 2 of VM formation by C32 melanoma cells, but had no effect on CHL-1 melanoma cells. This was also confirmed using scratch wound assays.

As highlighted in Chapter 1, interactions between cancer cells and platelets can be mediated by several surface expressed molecules. Gene expression analysis revealed that cell surface markers which allow for platelet-cancer interactions (e.g. *SELPLG* and *ITGB3*) were expressed at low levels in CHL-1 melanoma cells, intermediate levels in breast cancer cells, and high levels in C32 melanoma cell, which were confirmed with flow cytometry. This suggested that the inhibitory effect of platelets may depend on an interaction between platelets and cancer cells, and is further supported by the lack of effect of the inert platelet-sized beads (Chapter 3). The studies in Chapter 4 also revealed that supernatants from α-thrombin activated platelets also inhibited VM formation. This suggested that in addition to a direct interaction, soluble factors released by platelets can also inhibit VM formation. Furthermore, gene expression analysis revealed that the cancer cell line most sensitive to the effects of platelets, C32 melanoma, expressed very high levels of CD36 (TSP-1 receptor) in comparison to other cell lines which revealed negligible expression, and this was also confirmed using flow cytometric analysis. As discussed in detail in Chapter 4, TSP-1 is a potent angiogenesis inhibitor which signals through CD36 to inhibit EC tube formation (Chu, Ramakrishnan & Silverstein 2013).

To investigate the involvement of the TSP-1/CD36 axis in the inhibitory VM effect of platelets, siRNA knockdown of CD36 was performed on C32 melanoma cells which were then treated with platelet-derived TSP-1. This revealed two important findings; firstly, the knockdown of CD36 alone appeared to have inhibitory effects (although not statistically significant) on VM formation, wherein VM formation was attenuated when CD36 expression was reduced via siRNA. Secondly, whilst TSP-1 inhibited VM in C32 cells, TSP-1 had no further effect on C32 cells when CD36 was reduced. This suggests

that activation of the CD36/TSP pathway in C32 melanoma cell line is one of the mechanisms underpinning the platelet inhibitory effect on VM. Furthermore, this is supported by the resistance of the CHL-1 melanoma cell line, which expressed negligible levels of CD36 mRNA and protein.

Taken together, the studies in Chapter 3 and Chapter 4 revealed that VM formation is inhibited *in vitro* by platelets through both direct interaction and through contents released from platelets, and that this is regulated via the expression of CD36 by cancer cells and the release of anti-angiogenic factors by platelets (e.g. TSP-1). While further work needs to be done to confirm this, these studies have identified a novel pathway for the regulation of VM formation by cancer cells which is likely mediated through the contents released by activated platelets.

In Chapter 5, the *in vivo* role of platelets in VM was investigated using a xenograft mouse model of triple-negative breast cancer (TNBC) with and without impaired platelet function. Although a platelet function defect could not be confirmed in this study using qPCR (*Cox1* and *Cox2*) expression, as discussed in detail in Chapter 5, using the same or lower dose of aspirin (≤25mg/kg) via the same route of administration (oral), other studies have confirmed an inhibitory effect using TXA_2 ELISA (Carlson et al. 2013; Guillem-Llobat et al. 2016). While aspirin treatment significantly inhibited tumour growth within this model, an effect on the number of VM or EC-lined structures was not identified. Interestingly, histological analysis of the tumours did indicate more extensive micro-necrotic regions in tumours from mice following aspirin treatment, and this was supported by TUNEL staining. Furthermore, while no significant differences in metastases to distant organs (lungs, liver, spine, bones) were observed between the two groups of mice, fewer metastases to the lungs of aspirin treated mice were suggested. Finally, mice receiving

aspirin revealed elevated expression of genes *MMP2* and *MMP9*, which suggested an elevated ability of cancer cells to remodel the ECM in the tumour microenvironment.

6.2 **Implications of these findings**

The overarching aim of this study was to investigate the role of platelets in VM. Chapter 1 presented a review of the literature which highlights the well-established pro-angiogenic and pro-metastatic role of platelets in malignancy. Several cell-surface adhesion molecules are important in mediating platelet-cancer cell interactions, including P-selectin and $\alpha_v\beta_3$ integrin (Figure 1-8) (Gong et al. 2012a; Lonsdorf et al. 2012; Trikha et al. 2002). The interaction of platelets with cancer cells (tumour cell-induced platelet aggregation, TCIPA) is documented to enhance an aggressive phenotype in cancer cells, particularly through epithelial-to-mesenchymal transition (EMT) (Dovizio et al. 2013b; Labelle, Begum & Hynes 2011), a process which has also been implicated in VM formation by cancer cells (Chen et al. 2016; Liu et al. 2012; Ma et al. 2011; Yang et al. 2015). Therefore, it was hypothesised that platelets would promote VM formation by cancer cells. Although the studies presented in this work do not support the hypothesis, they have highlighted the important multi-functional role of platelets, in particular, their lesser known anti-tumorigenic potential (Italiano et al. 2008; Zaslavsky et al. 2010). The following section will discuss the implications of the studies presented in this thesis which have revealed that platelets inhibit VM by cancer cells through a direct interaction or through release of soluble factors, an effect which is dependent on the cancer phenotype (i.e. expression of particular cell surface markers), and finally, the implications of the *in vivo* findings.

6.2.1 **Interaction-specific platelet inhibitory effect of VM**

The formation of platelet-cancer cell aggregates in culture has been previously described, a phenomenon known as TCIPA (Heinmoller et al. 1996; Jurasz, Alonso-Escolano & Radomski 2004; Lindenmeyer, Legrand & Menashi 1997). The VM co-culture assay revealed that platelets were in close association with cancer cells, evident particularly in the C32 melanoma cell line. This is in line with previous research that both melanoma and breast cancer can induce TCIPA (Chang et al. 2015; Lindenmeyer, Legrand & Menashi 1997; Qi et al. 2014). Therapeutic strategies have used this to their advantage by producing nanovesicles which mimic the platelet surface that can be used for drug delivery to cancer cells in circulation (Uppal et al. 2014). Integrin $\alpha_v\beta_3$ mediates cancer cell-cancer cell interactions in the 3D co-culture system and inhibiting this interaction disrupts VM formation by melanoma *in vitro* (Vartanian et al. 2011b). Therefore, it is suggested that the addition of platelets to the cell culture system competitively binds to integrin $\alpha_v\beta_3$ through platelet GPIIb/IIIa and thus limits cancer cell-cancer cell interactions. A specific receptor-ligand interaction between platelets and cancer cells was supported by studies using inert-platelet sized beads in the VM assay, which did not inhibit VM formation. The studies have shown that platelets can inhibit VM formation via a direct interaction, possibly by competitively interacting with the cancer cells and thus inhibiting cancer cell-cell interactions that are necessary for VM to occur.

6.2.2 **The inhibitory effect of platelet-released soluble factors on VM**

Chapter 4 provided evidence that, in addition to a direct interaction, the anti-VM effect of platelets may also be mediated through the release of soluble angiogenesis inhibitors. Cancer cells have previously been identified to have high expression of tissue factor and thrombin (Heinmoller et al. 1995; Heinmoller et al. 1996; Mueller et al. 1992; Ueno et al. 2000). In accordance, gene expression analysis indicated that cancer cell lines used throughout this study may activate the coagulation pathway (tissue factor and prothrombin gene expression) to induce platelet aggregation, activation and release of platelet contents. Chapter 4 identified the potential involvement of the CD36 pathway in the platelet inhibitory effect on VM by the C32 melanoma cell line, which expressed high gene and protein levels of CD36, as previously documented by Silverstein and colleagues (Silverstein et al. 1992).

Thrombospondin-1, a ligand for CD36, is the most abundant protein contained in platelets (Burkhart et al. 2012) making up as much as 20% of the total released contents (Baenziger, Brodie & Majerus 1972). Via CD36, TSP-1 inhibits tube formation by ECs both *in vitro* and *in vivo* (Chu, Ramakrishnan & Silverstein 2013; Dawson et al. 1997; Jimenez et al. 2000; Koch et al. 2011). The studies in Chapter 4 have extended current knowledge through demonstrating that platelet TSP-1, signalling through CD36 receptor, mediates inhibitory effects on VM formation by C32 melanoma cells. As the C32 melanoma cell line expresses high CD36 on the cell surface, the anti-VM effects of platelet TSP-1 may also apply to other cancer cell lines which express CD36.

The studies in this chapter identified that CD36 (TSP-1-independent) may support VM formation by C32 melanoma cells. Previous studies have reported that CD36, via its ability to bind collagen, promotes cell adhesion to the ECM (Diaz-Ricart et al. 1993; Tandon, Kralisz & Jamieson 1989). Therefore, since the knockdown of CD36 in C32 melanoma cells reduced their ability to form VM, this suggested that CD36 is an important adhesion molecule in VM formation potentially by assisting cancer cells to adhere to the ECM in the initial stages of VM, and very recent studies in our laboratory investigating the role of CD36 in VM have recapitulated this result. While recent studies demonstrated that loss of CD36 function leads to continuous lipid accumulation and eventual lipotoxicity resulting in cell death through caspase-3 (Pascual et al. 2017), it is not likely that the *in vitro* VM tube formation is impacted by lipid content in the extracellular environment. The studies by Pascual investigating the function of CD36 in fat metabolism and malignancy should be considered in future prospective *in vivo* studies for investigating the role of CD36 in VM. Currently, there are is no literature on the role of CD36 in VM and these findings are the first to be reported. Furthermore, the current studies are in accordance with previous findings which have implicated TSP-1 and CD36 expression to be protective in malignancy (DeFilippis et al. 2012; Straume & Akslen 2005; Tsuchida et al. 1999; Yamaguchi et al. 2002). The current work demonstrates that the anti-angiogenic and tumorigenic effects of platelet TSP-1 content (Zaslavsky et al. 2010), also apply to VM formation by highly malignant cancer cells expressing CD36 and may suggest the CD36/TSP-1 axis as a novel therapeutic pathway with which to target VM formation by cancer cells.

6.2.3 **The *in vivo* role of platelets in VM?**

Preclinical and *in vivo* studies by others revealed that platelets can be found in close association with cancer cells in the tumour in a variety of cancer models including breast cancer, melanoma, colorectal, and others (Li et al. 2014a; Manegold et al. 2003; Qi et al. 2015; Stone et al. 2012; Yuan & Liu 2015), and that aspirin treatment (100mg/kg) does not alter the number of circulating platelets in mice (Kuznetsov et al. 2012). The studies in Chapter 5 have added to the current literature by demonstrating that platelets can be identified within the tumour microenvironment of mice receiving treatment of aspirin at 25mg/kg, a dose which is documented to render the platelets non-functional (Carlson et al. 2013; Guillem-Llobat et al. 2016). Furthermore, in support of previous studies which indicate a promotive role of platelets in malignancy (Ho-Tin-Noe et al. 2008; Li et al. 2014a; Palumbo et al. 2005; Qi et al. 2015), the aspirin treatment resulted in reduced tumour burden in animals.

To investigate the involvement of platelets in VM formation *in vivo*, immunohistochemical (IHC) staining for CD31 and periodic acid-Schiff (PAS) staining were utilised. No difference in VM formation between aspirin-treated and control mice was observed, despite evidence in Chapters 3 and 4 which suggested that platelets inhibit VM by cancer cells. However, as discussed in Chapter 5, the IHC and PAS staining are documented to have several limitations and these may impact on the outcome of the *in vivo* experiments (Buijs et al. 2004; Hendrix et al. 2001; Yang et al. 2016). Although histological analysis did indicate elevated apoptosis/necrosis in mice receiving aspirin treatment, whether this reflects an inhibition of the cancer cell-protective properties of platelets or is a result of inhibiting platelet activity remains to be determined. As discussed in detail in Chapter 5, tumour apoptosis/necrosis with low-dose aspirin treatment (25mg/kg) suggests the occurrence of hypoxia-induced cell death. This is based on

evidence that aspirin can inhibit mitochondrial function utilised by cancer cells to survive in the hypoxic tumour environment (Tewari et al. 2017; Vaupel, Mayer & Hockel 2004), as well as inhibiting the hypoxic response by being metabolised into inhibitors of HIF hydroxylase which attenuates HIF-1α degradation (Lienard et al. 2008).

Hypoxic conditions can also elevate *MMP2* and *MMP9* gene expression by cancer cells thus promoting spread and survival within the body (Choi et al. 2011; Lee et al. 2014; Zong et al. 2016). In accordance with this, it was observed that aspirin treatment did indeed elevate *MMP2* and *MMP9* gene expression by the cancer cells. It has also been documented that both *MMP2* and *MMP9* are elevated in VM, with *MMP2* cleaving laminin-5 γ2 chain into promigratory fragments γ2' and γ2x which promote VM formation by cancer cells (Hess et al. 2003; Seftor et al. 2001). While elevated gene expression of *MMP2* and *MMP9* in aspirin-treated mice suggests that the cancer cells have an elevated potential to degrade the ECM, other characteristic VM signature genes (*CDH5*, *EPHA2*, and *LAMC2*) although variable in their expression, showed no significant differences. This therefore highlights the need for further studies to confirm whether the elevated *MMP2* and *MMP9* levels may indicate progression towards a VM phenotype in aspirin-treated mice. For example, the progression towards a VM phenotype may be related to the expression of genes involved in EMT as this process has also been implicated to play a crucial role in VM formation (Liu et al. 2012; Sun et al. 2011; Yang et al. 2015). Genes of interest would include Twist, Snail, zinc-finger E-box binding homeobox 1 (ZEB1) and ZEB2.

As discussed in Chapter 1, the role of platelets in metastasis is well documented (Gay & Felding-Habermann 2011; Leblanc & Peyruchaud 2016; Menter et al. 2014). Chapter 5 investigated this in the breast cancer model following low-dose aspirin treatment, and this appears to be the first study to do so. While effects were not significant, analysis indicated

less metastases in the lungs of aspirin-treated mice compared with control mice, which is in accordance with previous studies carried out in models of 4T1 triple negative breast cancer, B16 melanoma, and HT29 human colon carcinoma (Coupland, Chong & Parish 2012; Guillem-Llobat et al. 2016; Li et al. 2014a; Lonsdorf et al. 2012). There was also some indication that aspirin caused more widespread liver metastases, which suggests that liver metastases may occur independently of the action of platelets in this *in vivo* model of breast cancer (Coupland, Chong & Parish 2012). Although the current model posed limitations for studying metastasis (discussed below), these studies in conjunction with others in the literature, have emphasised the complexity of platelet-dependent organ metastases and highlight the role of platelets in assisting metastases from the primary tumour to secondary distant sites.

6.3 **Limitations of the study and recommendations for future research**

The studies in Chapters 3 and 4 investigated the role of platelets in regulating VM *in vitro*, and the mechanisms involved. While these studies provide evidence that the platelet inhibitory effect may be dependent on surface expression of receptors which mediate platelet-cancer cell interactions, these findings would benefit from further functional studies. For example, assessing VM in co-culture with platelets following inhibition of platelet GPIIb/IIIa binding may limit interactions with cancer cell integrin $\alpha_v\beta_3$ (Trikha et al. 2002), without affecting cancer cell-cancer cell interactions (Vartanian et al. 2011b).

It is well documented that TCIPA results in release of platelet contents (Jurasz, Alonso-Escolano & Radomski 2004), and Chapter 4 revealed that VM can be inhibited via released contents from platelets activated with α-thrombin. While TSP-1 was identified as one of the mechanisms involved, other inhibitory mechanisms may be involved. For example, another anti-angiogenic factor contained within the α-granules of platelets is

angiostatin (Jurasz et al. 2003; O'Reilly et al. 1994). As discussed in Chapter 4, angiostatin can bind to integrin $\alpha_v\beta_3$, acting as an antagonist to inhibit the binding of other RGD sequence containing ligands (Tarui, Miles & Takada 2001). Therefore, angiostatin may contribute to the platelet anti-VM effect since cancer cells differ in their expression of integrin β_3 subunit. Currently, the effectiveness of angiostatin in blocking VM formation is unknown and remains to be elucidated. Several other questions also remain, in particular, whether the platelet-inhibitory effect on VM depends on the thrombin production from cancer cells expressing TF, thereby activating platelet PAR-4 receptor which is documented to induce release of angiogenesis inhibitors (Italiano et al. 2008; Ma et al. 2005).

The aim of Chapter 5 was to investigate the role of platelets in VM *in vivo* using a model of breast cancer with inhibited platelet function, however some limitations became evident. Firstly, the inhibitory effect of aspirin on *Cox-1* gene expression could not be confirmed using a published method via qPCR (Fidaleo, Berardi & Sartori 2008) (discussed in detail in Chapter 5), although other studies have shown a reduction in platelet function using the same or lower dose of aspirin (≤25mg/kg) as that used here (Carlson et al. 2013; Guillem-Llobat et al. 2016). Future studies would measure plasma TXB_2 levels for the validation of altered platelet function *in vivo*. Another consideration is to assess the ability to upregulate P-selectin in activated whole blood (cheek bleed of untreated and treated mice). In addition, the work would also benefit from the use of an antibody-induced (anti-GPIb) platelet-deficient mouse model (Yuan & Liu 2015) which would investigate VM formation in the absence of platelets. Secondly, the *in vitro* data may not be reflected *in vivo* due to the difference in models i.e. very controlled conditions (*in vitro*) versus a less controlled and more complex system (*in vivo*). While some components of the VM gene signature were elevated (*MMP2* and *MMP9*), others were

not (*CDH5*, *EPHA2*, and *LAMC2*), and although this may provide cancer cells with increased ability to remodel the ECM following aspirin treatment (as discussed previously), further studies (discussed above) are necessary to determine the precise role of platelets here. Finally, while the data indicated differences in metastasis following low-dose aspirin treatment *in vivo*, the effects were inconclusive. As discussed in Chapter 5, this observation may reflect the insufficient time available for secondary metastases to develop in this xenograft model. To overcome this limitation, an alternate model to investigate metastasis is essential, for example, resecting the primary tumour thereby allowing for sufficient time for disseminated cells from the primary tumour to develop into fully established and easily detectable metastases (Munoz et al. 2006). In addition to this, a syngeneic model is another future consideration to investigate the role of platelets in VM *in vivo*. For this work, an orthotopic xenograft model of TNBC was chosen over a syngeneic model as it provided the best representation of human cancer characteristics. Although this does not assess the involvement of the immune system in this setting. To overcome this, a consideration for future *in vivo* studies will be to investigate the role of platelets in VM using a syngeneic model (4T1 TNBC model and B16-F10). Despite that a syngeneic model will not be as representative of human tumours, this model will allow for assessing the important role of the immune system.

6.4 **Conclusion**

The current literature provides evidence that platelets promote tumour angiogenesis and metastatic progression in cancer. Based on this, the hypothesis of this study was that platelets would promote an alternative perfusion pathway utilised by cancer cells, namely VM. To investigate this, a number of aims were established: (i) to determine the role of platelets in VM *in vitro*; (ii) to determine the mechanisms involved; and (iii) investigating the role of platelets in VM using an *in vivo* model of breast cancer.

While the *in vivo* study revealed that aspirin treatment inhibited tumour burden, no difference in effect on VM formation was observed. Although conclusions cannot be made regarding the role of platelets in VM *in vivo*, an inhibitory role *in vitro* was found. The current work has revealed that platelets inhibit VM through a direct interaction or through the release of platelet contents, an effect which depends on the cancer phenotype and expression of cell surface markers. Furthermore, this investigation has identified for the first time the CD36/TSP-1 axis as a novel pathway in VM. This is the first report to demonstrate an involvement of platelets in VM formation, and it has highlighted the important multi-functional role of platelets, in particular, their anti-tumorigenic potential.

Appendix 1. Buffers and reagents

A1-1. Collagenase

194ml	Baxter Water for Irrigation
0.2g	Collagenase Type 1
2.2ml	100x CaCl2
2.2ml	MgSO4
22ml	10x HBSS with glucose
0.4ml	FBS

Ingredients were mixed and filtered through a 0.22μm filter, and stored for no more than 1 week at 4˚C.

A1-2. 0.9% Saline

900mL	MQ H_2O
9g	NaCl

Solution was Autoclaved.

A1-3. Human umbilical vein endothelial cell (HUVEC) Media

500mL	M199
100mL	Fetal Bovine Serum
7.5mL	Sodium Bicarbonate
10mL	HEPES buffer solution
5mL	Penicillin Streptomycin
5mL	Sodium Pyruvate
5mL	Non-essential amino acids

A1-4. HUVEC Wash

Prepared as HUVEC Media, but with 2% FBS only.

A1-5. Gelatin

750ml	Baxter Water for Irrigation
15.39g	Granular gelatin
32.6ml	FBS
19.6ml	7.5% NaHCO3
65.2ml	10x HBSS with Glucose

The water was warmed and gelatin added. The water and gelatin mixture was gently dissolved over low heat (without agitation), autoclaved then cooled to 4˚C to confirm it would set. The mixture was then re-thawed and remaining ingredients added.

A1-6. Heparin

0.05g	Heparin
10mL	1x PBS (Sterile)

Filter through a 0.2µm low-protein binding filter.

A1-7. 0.5M EDTA/PBS Stock

186g	EDTA
1L	1x PBS

With pellets of sodium hydroxide used to dissolve the EDTA. The pH was adjusted to 7.3 using hydrochloric acid. The final solution was passed through a 0.22µm filter and stored in a sterile bottle at RT.

A1-8. 0.01M EDTA/PBS

16ml	0.5M EDTA/PBS Stock
80ml	10x PBS (Sterile)

A1-9. 20x Phosphate buffered saline (PBS)

Up to 1L	MQ H_2O
160g	NaCl
4g	KCl
53.6g	Na2HPO4-7H_2O
4.8g	KH2PO4

Adjust pH to 7.4. Dilute to 1x with MQ H_2O.

A1-10. 1x PBS (Sterile)

1L	Baxter Water for Irrigation
100mL	10x PBS
800ml	Baxter Water for Irrigation

A1-11. 0.05% Trypsin

5mL	10x (2.5%) Trypsin (Gibco, Life Technologies, Carlsbad, CA, USA, Cat# 15090-046)
0.3mL	0.5M EDTA in PBS stock
25mL	10x PBS
219.7mL	Baxter Water for Irrigation

A1-12. 0.2% Trypan Blue

100mL	PBS
0.2g	Trypan Blue Dye

A1-13. Acid citrate dextrose (ACD) anticoagulant

2.5g	Tri-sodium citrate.2H20
1.5g	Citric acid
2.0g	Dextrose
100mL	Abtech water

The pH was adjusted to 4.5. The solution was store at 4°C

A1-14. 1M Calcium chloride ($CaCl_2$) solution

14.7 g	$CaCl_2$
100mL	MilliQ water

A1-15. HEPES-tyrodes buffer

8.0g	NaCl
0.2g	KCl
0.2g	MgCl2.6H20
1.0g	NaHCO3
0.057g	NaH2PO4.H20
0.9g	HEPES
1000mL	Abtech water

The pH was adjusted to 7.4, and solution was stored at 4°C

A1-16.Tyrode's platelet wash buffer

10mL	HEPES-Tyrode's buffer
178ul	Apyrase

Wash buffer was prepared on the day of the assay. The pH was adjusted to pH 6.5 using 1 N HCL. Buffer was kept at 37°C until used.

A1-17. Tyrode's platelet resuspension buffer

10mL	HEPES-Tyrode's buffer
17.8ul	Apyrase
30uL	1 M $CaCl_2$

Resuspension buffer was prepared on the day of the assay. The pH was adjusted to 7.4, if necessary. Buffer was kept at 37°C until used.

A1-18. FACS Fixative

1L	1x PBS (Sterile)
10mL	Formaldehyde
2g	Glucose
5mM	Sodium Azide

A1-19. TE Buffer

9.88mL	MQ H_2O
100µL	1M Tris Base
20µL	500mM EDTA

A1-20. 1.5% TAE-Agarose Gel

1.5g	Agarose
100mL	1x TAE Buffer

A1-21. 50x Tris-acetate-EDTA (TAE) Buffer

1L	MQ H_2O
242g	Tris Base
57.1mL	Glacial acetic acid
100mL	0.5M EDTA/PBS stock

Adjust pH to 8.0. Diluted to 1x for use.

A1-22. Aspirin

2.5mg	Acetylsalicylic acid
100µL	EtOH
900µL	Corn oil

Aspirin was dissolved in EtOH. The solution was then diluted with corn oil. Aspirin dose was made fresh daily.

A1-23. D-Luciferin (30mg/mL)

0.45g	D-Luciferin (potassium salt)
15mL	1xPBS

D-Luciferin was dissolved in PBS, solution was then passed through a 0.22µm filter. Aliquots were stored at -20°C. D-Luciferin dose (3mg/200µl) was prepared on day of injection by diluting stock 1:1 in 1xPBS. Each mouse received 3mg in 200µl injection.

A1-24. 10% buffered formalin

90mL	1xPBS
10mL	Formaldehyde (37-40%)

A1-25. Ammonia Water Solution (0.2%)

2mL	Ammonium hydroxide (concentrated)
1000mL	distilled water

A1-26. 3% Hydrogen peroxide

1mL	30% hydrogen peroxide (Chem supply)
9mL	1xTris buffered saline

A1-27. 0.5M TRIS buffer (10x)

60.6g	Tris hydrochloride
13.9g	Tris Base
1.0L	distilled water

Ingredients were dissolved in distilled water. The pH was adjusted to 7.6 at RT. Solution was stored at 4°C.

A1-28. 1.5M Saline solution (10x)

88.66g	Sodium chloride
1.0L	distilled water

A1-29. 1x TRIS buffered saline (TBS)

100mL	10x Tris buffer
100mL	10x Saline solution
800mL	distilled water
500µL	Tween 20

Solutions were mixed and shaken vigorously following addition of Tween 20. Solution was left to settle overnight before pH was adjusted to 7.6. TBS was used no later than 1 week.

A1-30. 0.1M Citric acid solution (Stock solution A)

21.01g	Citric acid monohydrate
1.0L	distilled water

A1-31. 0.1M Sodium citrate solution (Stock solution B)

29.41g	Trisodium citrate dehydrate
1.0L	distilled water

A1-32. Citrate antigen retrieval solution (CD41 IHC)

9mL	Stock solution A
41mL	Stock solution B

A1-33. 1% Hydrogen peroxide

1mL	30% hydrogen peroxide (Chem supply)
29mL	distilled water

A1-34. 0.1M Citrate buffer (CD31 IHC)

3.84g	Citric acid
200mL	distilled water

The pH was adjusted to 6.0.

A1-35. 10.9mM Citrate buffer (TUNEL)

1.05g	Citric acid
500mL	distilled water

The pH was adjusted to 6.5. Solution was made fresh for every experiment.

A1-36. 1M Tris-HCl

121.14g	Tris
800mL	distilled water

A1-37. 0.1M Tris-HCl

3g	Bovine serum albumin
20mL	Foetal bovine serum
10mL	1M Tris-HCl

Appendix 2. Supplemental figures

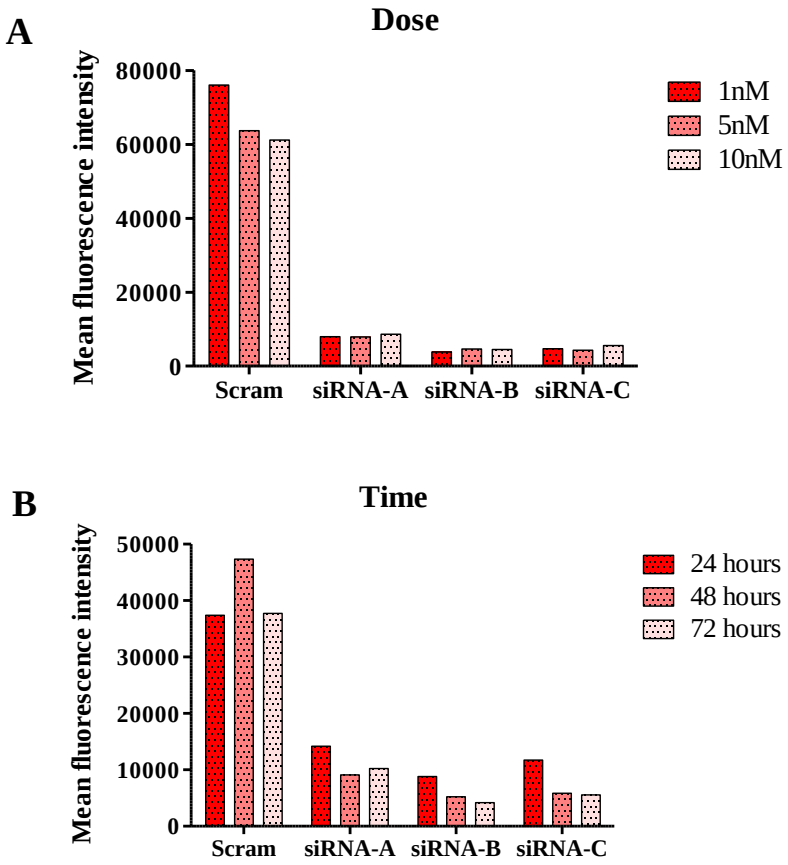


Figure A2-1. Dose and time optimisation of CD36 protein silencing via siRNA transfection.

(**A**) C32 melanoma cell line was transfected with 1nM, 5nM or 10nM of scrambled-siRNA (Scram) or CD36-targeting siRNA-A, siRNA-B, or siRNA-C, and mean fluorescence intensity was assessed at 48 hours via flow cytometric analysis. (**B**) C32 cells were transfected with 1nM of siRNA and mean fluorescence intensity was assessed at 24 hours, 48 hours, and 72 hours post transfection. Data are representative of a single experiment.

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