The Effect of Human Platelets on Lung Cancer Stem Cell Invasion

by

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ABSTRACT

Background: The cancer stem cell (CSC) theory suggests there are small populations of cancer cells that has stem cell-like characteristics, such as self-renewal. These CSCs are thought to be responsible for initiating new tumors following metastasis. Platelets have also been implicated in cancer metastasis, in part by stimulating cancer cell invasion. Moreover, studies show cancer cells can activate platelets. Stromal derived factor-1 alpha (SDF-1α), which may be secreted from activated platelets, can mobilize stem and CSCs via increased matrix metalloproteinase (MMP) expression. Thus, I hypothesize that SDF-1α from activated platelets can preferentially stimulate CSC invasion by binding to its receptor C-X-C chemokine receptor type 4 (CXCR-4) on CSC surfaces. Additionally, I hypothesize that the preferential stimulation of CSC invasion by platelets occurs due to an SDF-1α stimulated increase in CSC MMP production.

Methods: CSCs from the A549 lung carcinoma cell line were identified as Hoechst 33342-negative side population (SP) or identified and sorted using fluorescence activated cell sort (FACS) based on CD133-surface expression. Invasion assays using gelatin-coated Boyden chambers were used to compare the invasion of CSCs, non-CSCs, and total A549 population in response to collagen-aggregated human platelet releasates and quantified by flow cytometry and confocal microscopy. A549 CSC vs non-CSC MMP-2 and -9 protein levels were compared using gelatin zymography, and their MMP-2 mRNA levels were investigated using real time qPCR.

Results: A Hoechst-negative SP was identified within the A549 cell line and this SP was enriched with CD133-positive cells. Incubation of A549 cells with platelets or platelet releasates did not increase A549 cell CD133-surface expression, indicating platelets do not cause the conversion of CD133-negative into CD133-positive cells.

Collagen-aggregated platelet releasates preferentially stimulated invasion of both SP and CD133-positive cells. The CXCR-4 antagonist AMD3100 (10μM) failed to inhibit SP invasion, but inhibited total A549 invasion. AMD3100 decreased the invasion of CD133-positive cells. Although there was a trend toward reduced inhibition of total A549 invasion ($p = 0.07$), AMD3100 failed to cause a statistically significant decrease in this set of experiments.

A549 CD133-positive cells expressed higher basal MMP-2 levels than CD133-negative cells. 24-hour incubation with washed platelets increased MMP-2 protein in CD133-negative cells to CD133-positive cell levels, but did not change MMP-9 levels in either population. Real time qPCR revealed that CD133-negative cells increased MMP-2 mRNA expression to a greater extent than CD133-positive cells in response to incubation with platelets.

Conclusions: Within the A549 lung carcinoma cell line, subpopulations of cancer cells with CSC markers exist as the A549 SP is enriched with CD133-expressing cells. Aggregated platelet releasates preferentially stimulate invasion of A549 SP- and CD133-identified CSCs. This preferential stimulation of invasion likely involves platelet-derived SDF-1α signalling. CD133-positive cells have higher basal levels of MMP-2. Incubation with washed platelets increases MMP-2 levels in CD133-negative cells to CD133-positive cell levels. Further experiments are required to delineate the role of SDF-1α-CXCR-4 signalling in platelet-stimulated lung CSC invasion.

PREFACE

This thesis is an original work by Meng Jie Yan. Ethics approval for human platelet isolation was received from the University of Alberta Research Ethics Board for Dr. Jurasz's lab. Project name "NOS-based platelet subpopulations", No. Pro00029836, March 8, 2012. No part of this thesis has been previously published.

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AA - arachidonic acid

ABCB5 - ATP-binding cassette B5

ABCG2 - ATP-binding cassette sub-family G member 2

ALDH-1- aldehyde dehydrogenase 1

AML - acute myeloid leukemia

AMR - Ashwell-Morell receptors

ANGPT-1 - angiopoietin 1

ANOVA - analysis of variance

BAD - Bcl-2-associated death promoter

cAMP - cyclic adenosine monophosphate

cGMP - cyclic guanosine monophosphate

COX - cyclooxygenase

CSCs - cancer stem cells

CXCR-4 - C-X-C chemokine receptor 4

DAG - 1, 2-diacylgylcerol

DMEM - Dulbecco's Modified Eagle Medium

ECM - extracellular matrix

EDTA - ethylenediaminetetraacetic acid

ELISA - enzyme-linked immunosorbent assay

EMT - epithelial-mesenchymal transition

eNOS - endothelial nitric oxidase synthase

FACS - fluorescence activated cell sort

FITC - fluorescein

FBS - fetal bovine serum

FLT3 - fms related tyrosine kinase 3

GPCR - G-protein Coupled Receptor

GP - glycoprotein

HRP - horse radish peroxidase

IP3 - inositol 1,4,5-triphosphate

ITAM - immunoreceptor tyrosine-based activation motif

MET - mesenchymal epithelial transition

MMP - matrix metalloproteinase

MT-MMP - membrane type matrix metalloproteinase

NEU - neuraminidase

NO - nitric oxide

P38 MAPK - mitogen-activated protein kinase

PAK1 - P21 protein-activated kinase 1

PAR - protease-activated receptors

PDGF - platelet derived growth factor

PE - phycoerythrin

PI (3,4) P2 - phosphatidylinositol 3,4-bisphosphate

PI3K - phosphoinositide 3 kinase

PIP2 - phosphatidylinositol 4,5 bisphosphate

PIP3 - phosphatidylinositol 3,4,5-trisphosphate

PKC - protein kinase C

PLA_2 - phospholipase A2

PLC - phospholipase C

PLCγ2 - phospholipase Cγ2

PRP - platelet rich plasma

PTHrP - parathyroid hormone related peptide

PVDF - polyvinylidene difluoride

PYLL - potential years of life lost

RCF - relative centrifugal force

S1P - sphingosine 1 phosphate

SDF-1α - stromal derived factor-1alpha

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SP - Hoechst 33342-negative side population

TCIPA - tumor cell induced platelet aggregation

TGF-β - transforming growth factor beta

TGN - trans-Golgi network

TP receptors - thromboxane prostanoid receptors

tPA - tissue plasminogen activator

TPO - thrombopoietin

Trypsin-EDTA - trypsin-ethylenediaminetetraacetic acid

TXA_2 - thromboxane A2

uPA - urinary plasminogen activator

VEGF - vascular endothelial growth factor

vWF - von Willebrand factor

ZEB1 - zinc finger E-box binding homeobox 1

Introduction

1. Platelet Biology

2. Tumor Biology

3. Platelet and Tumor Interactions

1. Platelet Biology

1.1 Platelet Structure

Platelets, or thrombocytes, are small anucleated circulating blood cells whose primary function is to maintain hemostasis by forming clots at the site of an injury to arrest bleeding and aid in would healing. Numerous studies also provided evidence for the participation of platelets in other physiological processes such as angiogenesis as well as pathological conditions such as inflammation, myocardial infraction and cancer metastasis (Cangemi et al., 2014; Jurasz, Alonso-Escolano, & Radomski, 2004; Klinger & Jelkmann, 2002; Patzelt & Langer, 2012). Platelets are discoid-shaped, around 2-5 microns in size and are present in the vasculature at ~150 to 350 x10^6 platelets per milliliter of blood. Platelets contain a large number of granules that can be classified as either alpha-granules, dense-granules or lysosomes (Figure 1). While alpha-granules contains a variety of pro-coagulation factors, angiogenic factors, anti-angiogenic factors, growth factors and cytokines; dense-granules mostly contain small molecules such as serotonin and adenosine diphosphate (ADP) (Whiteheart, 2011). Upon activation, more than 300 different types of molecules are released by the platelets (Coppinger et al., 2004). These molecules are released when platelets undergo shape change and granule release in response to platelet activation with the help of the extensive actin and microtubule networks in the platelet cytoskeleton. Actin filaments anchor important membrane proteins, such as adhesive receptors, to the plasma membrane. α and β tubulin then forms a thick, ring-shaped microtubule net directly below and is tasked with maintaining the shape of platelets. When platelets are activated the microtubule ring contracts and shape change occurs when the actin cytoskeleton rearranges (Greenberg, 2012). Platelets have asymmetrical plasma membrane phospholipid distributions.

During the resting phase, phospholipids that support clotting are mostly hidden but are flipped to the outside by the integral membrane protein scramblase after activation occurs (Bevers, Comfurius, & Zwaal, 1983). Moreover, the plasma membrane of platelets is connected to an open canalicular system that forms the pathway for granule transportation and conducts the secreted contents of platelet granules to the outside (Escolar & White, 1991).

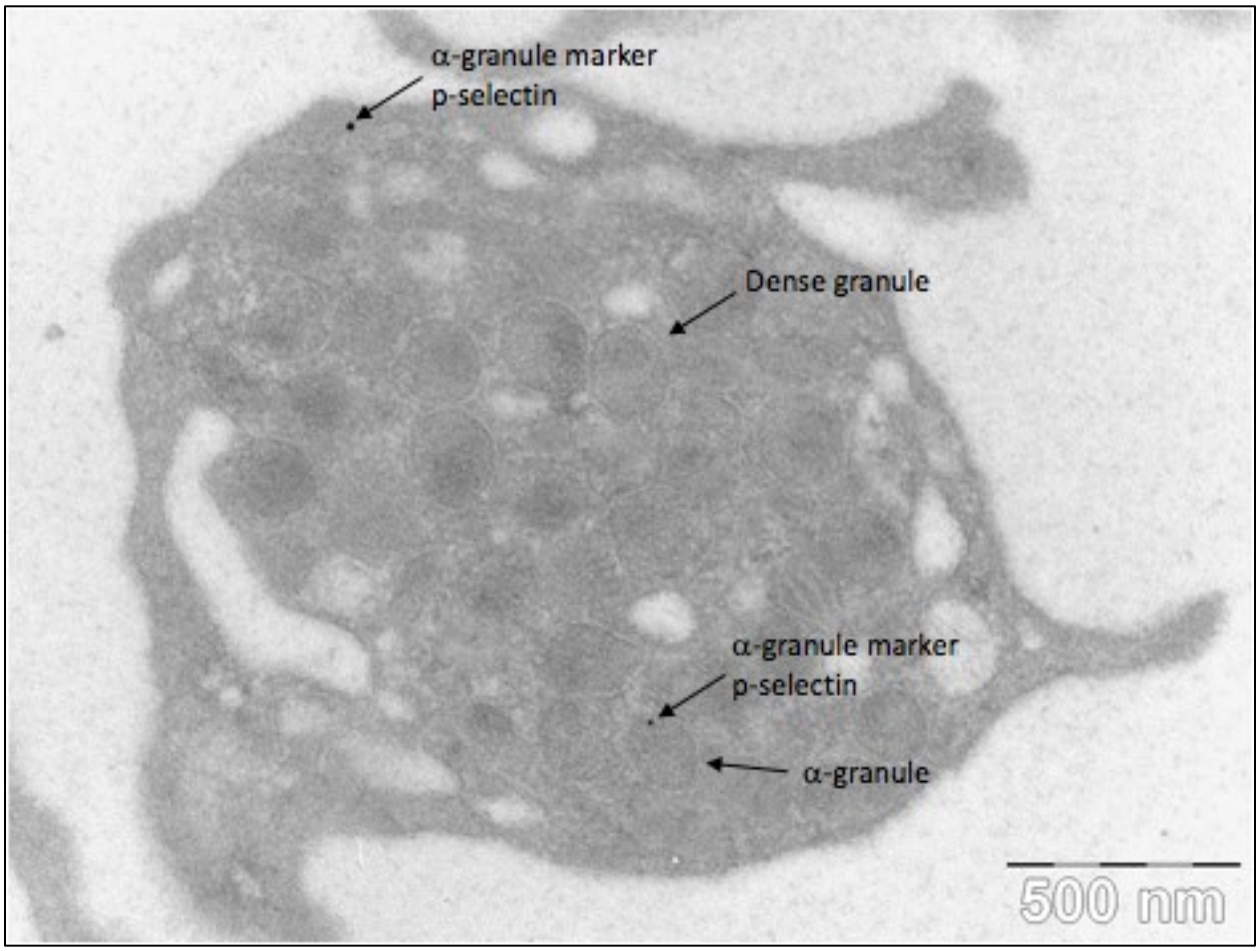


Figure 1: Electron microscope image of a platelet. (Unpublished, printed with permission from Dr. Paul Jurasz)

1.2 Platelet Life Cycle

Platelets are derived from the cytoplasm of the large bone marrow cells, megakaryocytes (Pease, 1956). Megakaryocytes are formed from a fms related tyrosine kinase 3 (FLT3)-negative hematopoietic stem cell derived lineage of erythroid progenitor cells (Pang, Weiss, & Poncz, 2005). Upon stimulation by the serum growth factor thrombopoietin (TPO), megakaryocytes disassemble at one pole to extend small portions of their cytoplasm and form pseudopods. These pseudopods eventually stretch to form long protrusions known as proplatelets (Richardson, Shivdasani, Boers, Hartwig, & Italiano, 2005). As proplatelets mature, the microtubules within the proplatelet arms loop around themselves, forming tips where the proplatelets to branch out. The rapid polymerization of the microtubules further elongates the proplatelets to eventually protrude into the vascular lumen of the bone marrow (Hartwig & Italiano). High concentrations of sphingosine 1 phosphate (S1P) in the blood stimulate the proplatelets to release individual platelets from their tips, thus forming anucleated mature platelets (L. Zhang et al., 2012). Due to the anucleated nature of platelets, most of the proteins contained in the platelets are received as packed granules from the parent megakaryocytes (Italiano, Lecine, Shivdasani, & Hartwig, 1999).

Within humans, mature platelets circulate in the vasculature for up to 10 days before they are cleared from the body though the liver and spleen (Dormehl, Kilian, Maree, & Jacobs, 1989). Platelet clearance involves platelet desialylation – the removal of sialic acid from platelet surfaces. The major target of platelet desialylation is GP Ibα, which is a subunit of the vWF receptor, although GP V is also a known target of desialylation. The major enzyme involved in

platelet desialylation is lysosomal neuraminidase 1 and 3 (NEU1&3) (Jansen et al., 2012). Desialylation exposes the galactose and β-N-acetyl-D-glucosamine (βGN) residues on the GP Ibα subunit and allows the formation of GP Ibα clusters, which can induce apoptosis in platelets (van der Wal, Du, et al., 2010; van der Wal, Verhoef, et al., 2010). Platelet survival is regulated by an internal apoptotic clock which is dependent of the balance of the pro-survival protein Bcl-xl and the pro-apoptotic proteins Bak and Bax (Leytin, 2012; Mason et al., 2007). The clustering of GP Ibα subunits leads to the association of GP Ibα with the adaptor protein 14-3-3, which normally binds the Bcl-2-associated death promoter (BAD) protein (van der Wal, Du, et al., 2010). This leads to the activation of BAD and the deactivation of Bcl-xl via the formation of the Bad-Bcl-xl heterodimers. The result is the activation of the pro-apoptotic proteins Bax and Bak and ultimately the activation of caspase 9 and the intrinsic apoptotic pathway (van der Wal, Du, et al., 2010; X. H. Zhang et al., 2015). The desialylated and apoptotic platelets are cleared by the hepatocytes via binding to hepatic Ashwell-Morell receptors (AMR) (Rumjantseva et al., 2009). Importantly, this binding of desialylated platelets to AMRs induce the release of TPO through the activation of the JAK2/STAT3 pathway. As hepatocytes are the major source of TPO, clearance of platelets by AMR initiates an important feedback mechanism that regulates platelet production (Grozovsky et al., 2015).

1.3 Platelet Activation & Aggregation

Platelet's hemostatic functions are initiated within 1-3 seconds after injury to a blood vessel and generally involves three distinct steps: formation of the platelet plug, consolidation of the platelet plug, and finally, fibrinolysis after the injury has healed. Circulating platelets adhere to the subendothelium exposed during injury through the interactions between platelet GP Ib-V-IX and the adhesion molecule vWF on exposed the subendothelium (Nuyttens, Thijs, Deckmyn, & Broos, 2011). This anchors the platelet enough so it can interact with exposed collagen in the subendothelium via GP VI and integrin α2β1 (Heemskerk et al., 1999). Platelet interaction with collagen causes secretion of soluble agonists such as thrombin, epinephrine, ADP and serotonin that further induces platelet activation in an autocrine manner (Brass, 2003; N. Li, Wallen, Ladjevardi, & Hjemdahl, 1997; Puri & Colman, 1997; Roberts, McNicol, & Bose, 2004; Spalding et al., 1998). The activation of platelets is accompanied by increased intracellular calcium concentrations which results in the phosphorylation of myosin light chains in the platelet cytoskeleton. Myosin light chains can then interact with the platelet's actin network to traffic granules and ultimately leads to platelet granule exocytosis and induces platelet shape changes that are characteristics of platelet activation (Aslan, Itakura, Gertz, & McCarty, 2012; Daniel, Molish, Rigmaiden, & Stewart, 1984). These activated platelets can then recruit and induce the activation and adhesion of the surrounding platelets through either direct contact or through the release of more soluble agonists which results in the formation of a loose platelet plug.

The consolidation of the platelet plug involves the activation of the coagulation cascade and the formation of the fibrin matrix. There are two pathways within the coagulation cascade.

The extrinsic pathway involves tissue factor, which is released from the damaged endothelium (Mackman, 2009). The intrinsic pathway, on the other hand, involves the activation of factor XI by factor XII, and ends in the formation of the factor IX-factor VIII tenase complex (Gailani & Renné, 2007). However, both pathways result in the activation of factor X, allowing it to form the crucial prothrombinase complex that cleaves prothrombin to thrombin. Thrombin is required to cleave fibrinogen to the insoluble fibrin, which then forms a fibrin net with the help of the transglutaminase factor XIII to trap more platelets as well as other blood cells, and finally result in the formation of a stable platelet plug/thrombus (Palta, Saroa, & Palta, 2014; Wolberg & Campbell, 2008) (Figure 2).

At the end of the process, the major fibrinolytic enzyme plasmin dissolves the fibrin clot in a process known as fibrinolysis (Cesarman-Maus & Hajjar, 2005; Stafford, 1964). Active plasmin is cleaved from the inactive plasminogen by tissue plasminogen activator (tPA) and urokinase (uPA). Moreover, fibrin itself can bind both plasminogen and tPA, thereby increasing the production of plasmin and enhancing fibrinolysis locally (Wolberg & Campbell, 2008).

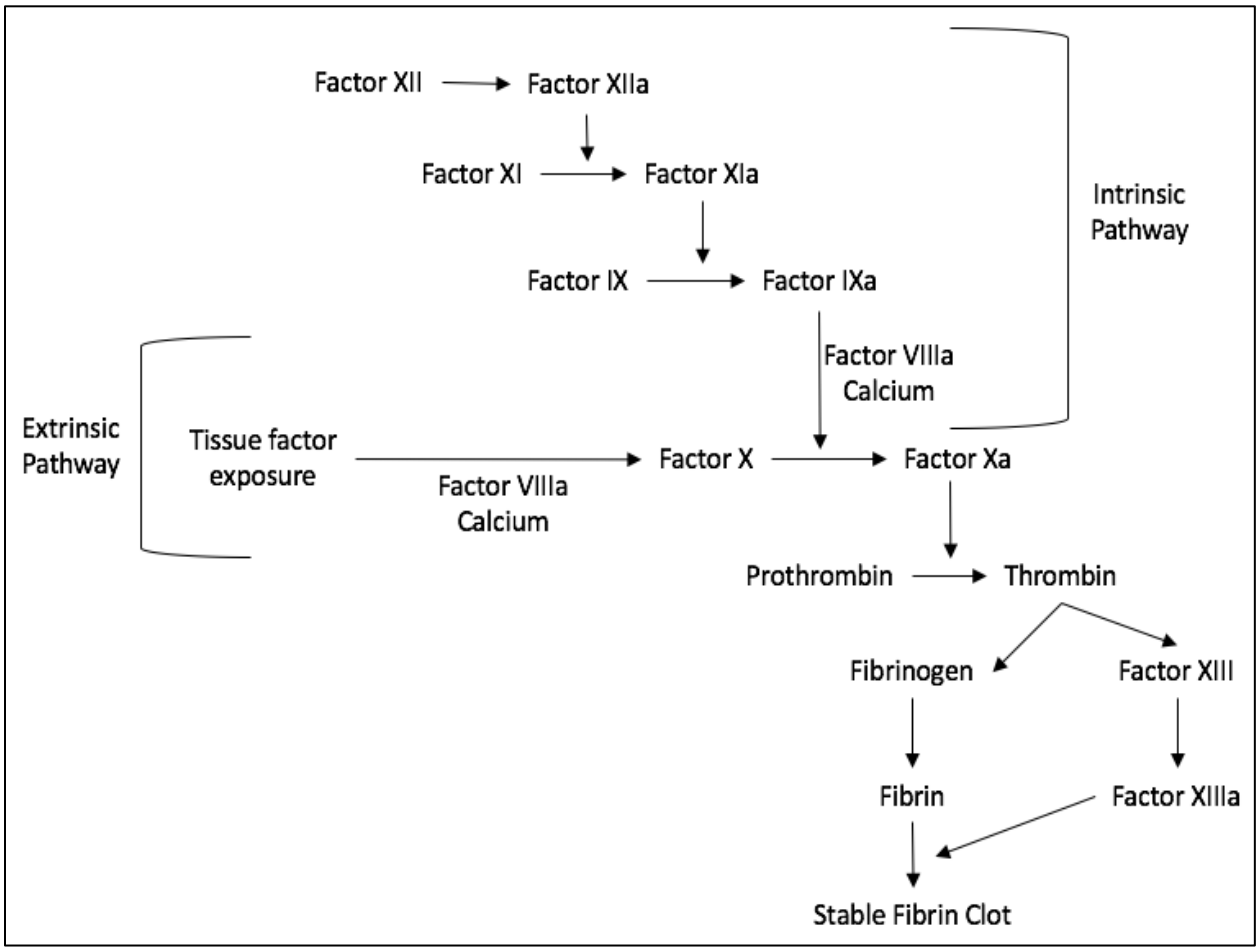


Figure 2: Coagulation pathways.

1.4 Platelet Activation stimulators and Inhibitors

Platelet agonists can be classified into either G-protein Coupled Receptor (GPCR) activating agonists or non-GPCR activating agonists, depending on the activation pathway of their receptors (Table 1) (Figure 3). GPCR activating agonists include thrombin, thromboxane A_2 (TXA_2), ADP serotonin and epinephrine (Baramova et al., 1997; Stefan Offermanns, 2006; Shattil, Budzynski, & Scrutton, 1989). When these agonists bind to their respective GPCR on the platelet surface, the associated G protein activates the phospholipase C (PLC) signalling pathway. This leads to the hydrolysis of downstream phospholipid phosphatidylinositol 4,5 bisphosphate (PIP_2) into the "secondary messengers" inositol 1,4,5-triphosphate (IP_3) and 1.2-diacylgylcerol (DAG) (Neer, 1995). IP_3 stimulates the phosphorylation of the myosin light chains by increasing intracellular calcium concentrations. Myosin light chains can then interact with the platelet's actin network to traffic granules and induce platelet shape change. DAG on the other hand, activates protein kinase C (PKC), and eventually leads to the secretion of the granules in a calcium dependent manner (Purvis, Chatterjee, Brass, & Diamond, 2008; Walker & Watson, 1993). Importantly, the activation of platelets also causes the conformation change of GP IIb/IIIa, which is required for its binding to fibrinogen (Ginsberg, Loftus, & Plow, 1988).

Table 1: Common platelet activation agonists and their mechanisms.

Agonist	Platelee-Activaing Mechanism	Reference
Thrombin	GCPR, PAR-1 & -4 receptor activation	Kahn et al., 1998
Thromboxane A2	GCPR, TP recpetor activaiton	Paul, Jin & Kunapuli, 1999
ADP	GCPR, purinergic receptor activation	Murugappa & Kunapuli, 2006
Serotonin	Indirectly activate platelets via ADP	Li et al., 1997
Epinephrine	Induction of platelet firbinogen receptor expression	Shattil, Budzynski & Scrutton, 1989
Collagen	Integrin α2β1 & GP VI receptor activation	Roberts, McNicol & Bose, 2004
MMP-2	Potentiation of PI3K activation	Falcinelli et al., 2005

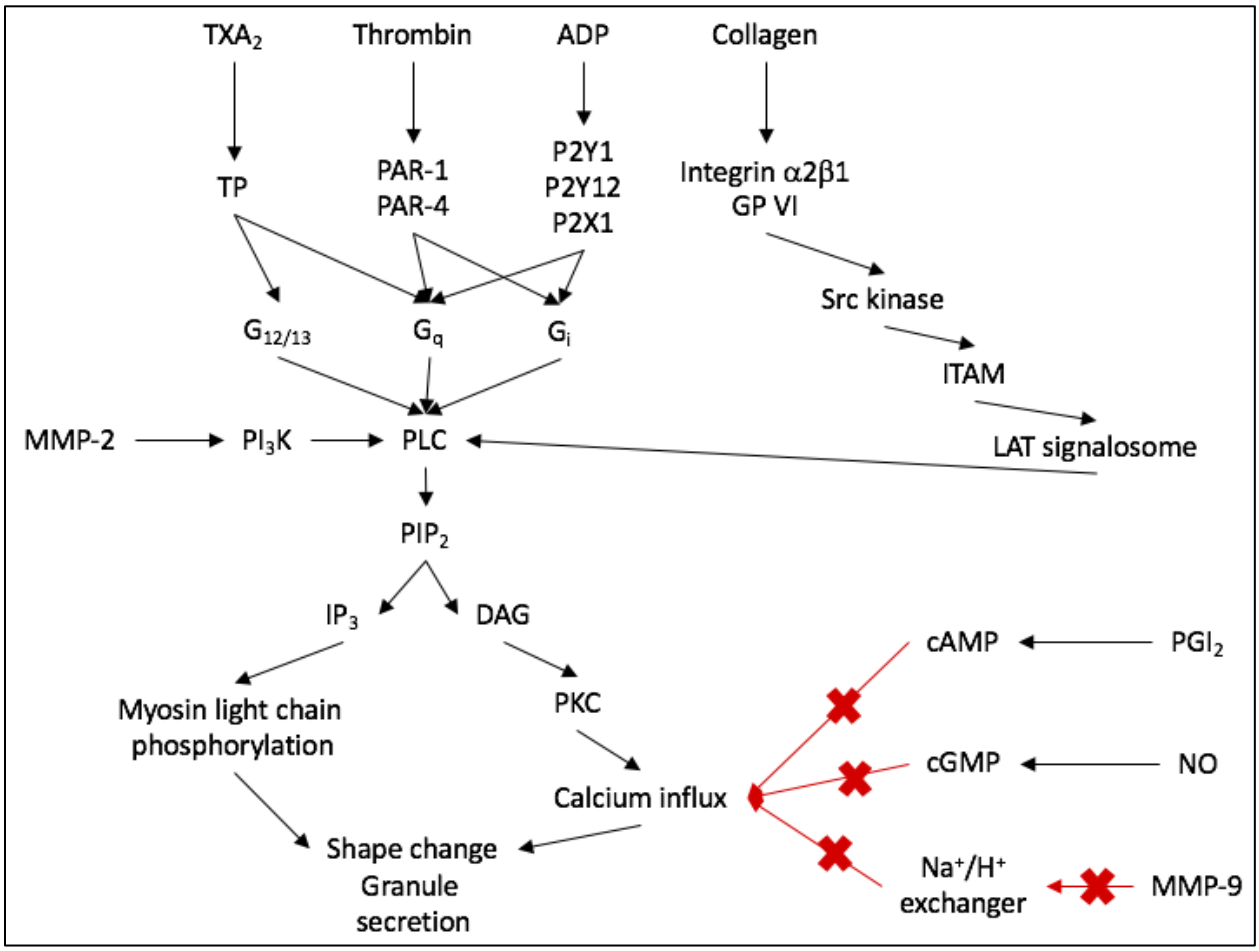


Figure 3: Common platelet activation stimulators, inhibitors and their mechanisms.

Thrombin is an endolytic serine protease and is considered the most potent platelet agonist. It works by activating a family of GPCRs known as protease-activated receptors (PAR) (Kahn et al., 1998). More specifically, thrombin activates the PAR-1 and PAR-4 receptors on human platelet surfaces by the cleavage of the N-terminus extracellular domain. This reveals a special amino acid sequence that tethers intramolecularly to the body of the receptor to initiate signalling events, including shape change, the release of other platelet agonists from platelet granules, mobilization of adhesion proteins and GP IIb/IIIa activation, that ultimately ends in the consolidation of the platelet plug (Brass, 2003; Vu, Hung, Wheaton, & Coughlin, 1991).

TXA_2 is another potent platelet agonist that activate platelets via GPCR-dependent pathways. The generation of TXA_2 involves the release of arachidonic acid (AA) from platelet membrane phospholipids through the actions of phospholipase A2 (PLA_2). AA is then oxidized to prostaglandin H2 (PGH_2) by cyclooxygenase (COX) -1 enzymes. Lastly, PGH_2 is converted to TXA_2 by thromboxane synthase (Samuelsson, 1987). TXA_2 activates the G protein-coupled thromboxane prostanoid receptors (TP receptors) and acts through the $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$ subunits. This then leads to the activation of the PLC pathway and the formation of IP_3 and DAG (Djellas, Manganello, Antonakis, & Le Breton, 1999; Knezevic, Borg, & Le Breton, 1993; S. Offermanns, Laugwitz, Spicher, & Schultz, 1994; Shenker, Goldsmith, Unson, & Spiegel, 1991; Ushikubi et al., 1989; Ushikubi, Nakamura, & Narumiya, 1994). Although TXA_2 can directly activate $G\alpha_q$ and $G\alpha_{12/13}$, one study suggests TXA_2 mediated $G\alpha_i$ requires another GPCR, P2Y12, activation by ADP beforehand (Paul, Jin, & Kunapuli, 1999). Moreover, by binding to Gαi subunits, TXA_2 frees the Gβγ subunits which allows it to bind and activate phosphoinositide 3 kinase (PI_3K). This then leads to the activation of the Akt pathway via phosphatidylinositol 3,4-

bisphosphate (PI(3,4)P2) and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Rittenhouse, 1996; Woulfe, 2010). PI3K itself is also know to play a role in thrombin, ADP and vWF stimulated platelet activation (Woulfe, 2010).

ADP is the first known and the first low-molecular weight platelet agonist identified (Gaarder, Jonsen, Laland, Hellem, & Owren, 1961). Importantly, although ADP is considered a weak platelet agonist, ADP released from platelet storage in response to other platelet agonists, such as thrombin, is vital in propelling a second wave platelet aggregation that recruits additional platelets (Puri & Colman, 1997). The GPCRs important in ADP-mediated platelet aggregation include the purinergic receptors: P2Y1, P2Y12 and P2X1. While P2Y1 mediates its effect through $G\alpha_q$ subunits, P2Y12 receptors mediate its effect through $G\alpha_i$ subunits. However, P2Y12 receptors are the ones responsible for the potentiation of the platelet activating effects of other platelet agonists, such as thrombin and TXA_2. Lastly, P2X1 receptors leads to the activation of calcium channels and the influx of calcium then results in platelet shape change and granule secretion (Murugappa & Kunapuli, 2006). Interestingly, ADP can also induce PLA_2 activation through the ADP receptors P2Y1 and P2Y12, and integrin αIIbβ3 activation. This then leads to cleavage of AA from platelet membranes and eventually TXA_2 production (J. Jin, Quinton, Zhang, Rittenhouse, & Kunapuli, 2002).

Collagen is an example of a non-GPCR activating platelet agonists. There are two major collagen receptors on the platelet surface: integrin α2β1 and GP VI. The first interaction between collagen exposed during injury and platelets is through vWF immobilized in the collagen and GP Ib-V-IX expressed on the platelet surface. However, the interaction between vWF and GP Ib-V-

IX is weak and transient, and will only result in the rolling, but not the firm adhesion, of platelets along the exposed subendothelial surface (Doggett et al., 2002; Savage, Saldivar, & Ruggeri, 1996). During rolling, platelets are able to initiate further contact with collagen through GP VI. Although GP VI have relatively low affinity for collagen, its importance lies in that it is able to signal the activation of integrin α2β1 in addition to inducing the release of the soluble agonists ADP and TXA_2 (B. Nieswandt et al., 2001; Savage et al., 1996). Integrin α2β1 is the main direct collagen adhesion receptor expressed on the platelet surface that is necessary for the firm adhesion of platelets to collagen. Although integrin αIIbβ3 can also mediate firm adhesion of platelets and stop rolling, it does not bind collagen directly but rather to vWF immobilized in collagen (Ni & Freedman, 2003). Integrin α2β1 exists in a low affinity conformation and requires activation to the high-affinity conformation via GP VI and other platelet agonists including ADP, TXA_2 and thrombin (Stephanie M. Jung & Moroi, 2000). Moreover, the ADP and TXA_2 released as a result of GP VI signalling can further activate platelets independent of GP VI (Kuijpers et al., 2003; Bernhard Nieswandt & Watson, 2003). It has been suggested that the downstream conduction of GP VI signals is dependent on tyrosine phosphorylation. Upon GP VI binding to collagen, the Src protein kinases Fyn and Lyn phosphorylates the Tyr residue of the immunoreceptor tyrosine-based activation motif (ITAM). The activated ITAM is then able to activate the tyrosine kinase Syk, which will result in the activation of the LAT signalosome (Boulaftali, Hess, Kahn, & Bergmeier, 2014). Among other components of the signalosome, phospholipase Cγ2 (PLCγ2) is able to cleave PIP_2 into DAG and IP_3. These two proteins can then activate the PKC pathway and induce an increase in intracellular calcium, respectively (S. M. Jung & Moroi, 2008; Roberts et al., 2004).

Another group of factors that is important in platelet activation are the matrix metalloproteases (MMPs). Pro-MMPs such as pro-MMP- 1, -2, -3, -9 and -14 are found in human platelets, and they are activated by proteolytic cleavage before being secreted upon platelet activation, apart from MMP-14 which is membrane-bound. More importantly, these released MMPs were found to affect platelet activation and aggregation. (Fernandez-Patron et al., 1999; Galt et al., 2002; Kazes, Elalamy, Sraer, Hatmi, & Nguyen, 2000; Sawicki, Salas, Murat, Miszta-Lane, & Radomski, 1997). Of these, the collagenase MMP-1 and the gelatinase MMP-2 was found to have platelet activating abilities. MMP-1 was shown to participate in collagen-mediated platelet activation (Trivedi et al., 2009). In addition to activating platelets via the ADP/TXA_2 pathway as described above, collagen can also activate platelets though P38 mitogen-activated protein kinases (p38 MAPK) pathway. Specifically, Rho-GTPases such as Rac and Cdc42 which are also activated by collagen, can activate p38 via downstream effector p21 activated kinase (PAK-1) and result in the activation of integrin αIIbβ3 (Z. Li, Zhang, Feil, Han, & Du, 2006). Previously, activation of p38 in platelets was found to be stimulated by either thrombin or vWF (Begonja et al., 2007; Canobbio, Reineri, Sinigaglia, Balduini, & Torti, 2004). However, newer evidence found the activation of this pathway can also be caused by the cleavage of PAR-1 receptors by activated MMP-1. Interestingly, MMP-1 cleaves PAR-1 at a site distinct from the thrombin cleavage site (Trivedi et al., 2009).

MMP-2 is released by platelets after activation by either collagen or thrombin, and this release of MMP-2 is important for the mediation of a TXA_2 and ADP independent platelet aggregation pathway. Inhibition of MMP-2 using the endogenous MMP inhibitor tissue inhibitors of matrix metalloproteinases (TIMP-2) or the broad spectrum MMP inhibitor

phenanthroline inhibited collagen induced platelet aggregation (Sawicki et al., 1997). Similarly, in animal models, mice with inactive MMP-2 genes showed decreased thrombus formation in response to collagen. Moreover, thrombus formation downstream of arterial injury sites is dependent on platelet-derived MMP-2 (Momi et al., 2009). During platelet activation, pro-MMP-2 in the cytosol translocates to the platelet surface membrane where it is activated in the MT-MMP-1/TIMP2/MMP-2 trimolecular complex (Kazes et al., 2000; Sawicki et al., 1998). Once activated, MMP-2 amplifies platelet aggregation and secretion in response to agonists such as collagen and thrombin. MMP-2 induced increases in the production of PI3K and the mobilization of intracellular calcium (Falcinelli, Guglielmini G Fau - Torti, Torti M Fau - Gresele, & Gresele, 2005). These events are a result of the potentiating effect of MMP-2 on the activation of PI3K, which is an important signalling molecule involved in the platelet aggregating effects of GPCR activating agonists such as thrombi and ADP (Falcinelli et al., 2005; Woulfe, 2010).

In addition to platelet agonists, platelet inhibitors can be found either secreted from platelets (e.g. MMP-9), or generated by nearby cells (e.g. nitric oxide (NO)) (Table 2). MMP-9 is the second known gelatinase after MMP-2. Similar to MMP-2, MMP-9 is stored in platelet cytoplasm in the pro-form. Although resting platelets release pro-MMP-9 basally, maximal release of MMP-9 only occur at sub-threshold levels of platelet aggregation (Fernandez-Patron et al., 1999). Once released, MMP-9 is activated by proteases such as plasmin, elastase and tissue kallikrein (Baramova et al., 1997; Desrivieres et al., 1993; Vissers & Winterbourn, 1988). Interestingly, MMP-9 is able to inhibit platelet activation induced by a variety of agonists including collagen, thrombin and AA. Furthermore, this inhibition is dependent on the enzymatic activity of MMP-9 (Fernandez-Patron et al., 1999; Sheu et al., 2004). MMP-9 was found to

inhibit ADP-mediated platelet activation, as well as ADP release induced by other platelet agonists such as collagen and thrombin. This lead to the inhibition of downstream inositol phosphate formation and an inhibition of intracellular calcium influx in response to collagen stimulation (Sheu et al., 2004). MMP-9 inhibition of platelet intracellular calcium mobilization is likely through inhibition of the Na+/H+ ion exchanger (Y. M. Lee, Lee, Shen, Hsiao, & Sheu, 2006).

Table 2: Common platelet activation antagonists and their mechanisms.

Antagonist	Platelet-Activating Mechanism	Reference
Prostacyclin	Induction of platelet cAMP production	Moncada, Gryglewski, Bunting, & Vane, 1976
Nitric Oxide	Induction of platelet cGMP production	Moncada & Higgs, 2006
MMP-9	Inhibition of platelet intracellular calcium mobilization	Y. M. Lee, Lee, Shen, Hsiao, & Sheu, 2006)

Another endogenous platelet inhibitor is prostacyclin. It is a potent platelet inhibitor produced by endothelial cells and is a product of AA metabolism. AA produce PGH_2 as a product of oxidation by the COX enzymes. Prostacyclin is then formed from PGH_2 by the actions of prostacyclin synthase (Samuelsson, 1987). Prostacyclin inhibits platelet activation by activating platelet adenyl cyclase, and thus increasing platelet synthesis of cyclic adenosine monophosphate (cAMP) in platelets (Moncada, Gryglewski, Bunting, & Vane, 1976). Increased concentrations of cAMP leads to increased phosphorylation of the cAMP dependent protein kinase A I and II (PKAI & PLAII) and result in the deactivation of Ras and RhoA (Kawata et al., 1989). Ras and Rho pathways are important in the mobilization of intracellular calcium, which drives the modification of platelet cytoskeleton upon platelet activation. Thus inhibition of Ras and Rho can lead to the inhibition of platelet activation (Smolenski, 2012). Interestingly, although platelets themselves do not produce prostacyclin, endothelial cells can utilize AA and downstream endoperoxidases produced by platelets in their formation of prostacyclin, suggesting the presence of an auto-regulatory pathway of AA-mediated platelet activation in platelets (Marcus, Weksler, Jaffe, & Broekman, 1980).

In a pathway parallel to the release of prostacyclin in endothelial cells, NO is synthesized by endothelial nitric oxide synthase (eNOS). Endothelial derived NO not only prevent platelet aggregation, but also prevent platelet adhesion to the endothelium (Moncada & Higgs, 2006; M. W. Radomski, Palmer Rm Fau - Moncada, & Moncada, 1987a, 1987c). NO-mediated inhibition of platelet adhesion is dependent on the production of 3',5'-cyclic guanosine monophosphate (cGMP) in platelets (M. W. Radomski, Palmer Rm Fau - Moncada, & Moncada, 1987d). The increase in platelet cGMP was also found to be important for NO-mediated inhibition of platelet

aggregation (M. W. Radomski, Palmer Rm Fau - Moncada, & Moncada, 1987b). cGMP inhibits platelet function through its downstream target protein kinase G (PKG) to inhibit PKC activation, and as a result, the inhibition of IP_3 and intracellular calcium mobilization (Durante, Kroll Mh Fau - Vanhoutte, Vanhoutte Pm Fau - Schafer, & Schafer, 1992; Nakashima S Fau - Tohmatsu, Tohmatsu T Fau - Hattori, Hattori H Fau - Okano, Okano Y Fau - Nozawa, & Nozawa, 1986). In addition, cGMP can also induce the phosphorylation, and thus inhibition, of TXA2 receptors to inhibit platelet aggregation (G. R. Wang, Zhu Y Fau - Halushka, Halushka Pv Fau - Lincoln, Lincoln Tm Fau - Mendelsohn, & Mendelsohn, 1998). Moreover, a L-arginine/NO pathway was identified in platelets (M. W. Radomski, Palmer Rm Fau - Moncada, & Moncada, 1990b). L-arginine, the precursor of nitric oxide, inhibited agonist, such as collagen, ADP and AA-induced aggregation of washed platelets and induced cGMP production in these platelets, suggesting the presence of NOS in platelets and its potential role in regulating platelet aggregation (M. W. Radomski, Palmer Rm Fau - Moncada, & Moncada, 1990a).

In addition to the large number of factors that can affect platelet function, platelet themselves contain and release numerous factors that affect other cells. The role of platelet factors in mediating tumor angiogenesis and metastasis has long been an area of interest for researchers. For example, factors like vascular endothelial growth factor (VEGF), angiopoietin-1 (ANGPT-1), MMP-2 has strong implications in tumor angiogenesis (Yan, Lesyk, Radziwon-Balicka, & Jurasz, 2014). Other factors such as, platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β) and MMP-9 has been extensively studied in their participation in cancer metastasis (Borsig, 2008). Recently, stromal derived factor-1alpha (SDF-1α) has been receiving attention for its cancer cell mobilizing effect (Tamamis & Floudas, 2014).

2. Tumor Biology

2.1 Cancer Statistics

Cancer is a collection of complex genetic diseases. Their initiation requires a multi-step process in which multiple mutations and lesions needs to be accumulated overtime before a normal cell can become malignant. Thus, although cancer can happen at a young age, the occurrence of many types of cancers have high correlations with aging (Renan, 1993). The accumulation of mutations eventually produces malignant cells that grow uncontrollably, have unlimited life-span, are often resistant to treatment, can spread to other parts of the body, and can reappear after their removal (Hanahan & Weinberg, 2011). The mutations, which can be caused by various genetic and environmental factors, often lead to the over-expression and activation of proto-oncogenes and the loss of expression and function of tumor suppressor genes (Hanahan & Weinberg, 2000, 2011). Proto-oncogenes are genes that can cause cancer when they undergo mutation and become oncogenes, and they are often highly expressed in cancer. Oncogenes are important in the development of cancer in that they often cause the dysregulation of key factors that govern cell life cycle, and thus mutations in these cells can produce cancer cells with uncontrollable proliferation and are able to evade physiological signals that induce cell death (Weinstein & Joe, 2006). Examples of oncogenes include epidermal growth factor receptors (EGFR) that regulate effects mediated by epidermal growth factor (EGF) and the kinase insert domain receptor gene (KDR), which encodes the receptor for VEGF - vascular endothelial growth factor receptor 2 (VEGFR-2) and is an important factor in driving tumor angiogenesis (Gazdar, 2009; W. Wu, Shu, Hovsepyan, Mosteller, & Broek, 2003).

Currently, cancer is the leading cause of death in Canada, accounting for 29.9% of all death, followed by cardiovascular diseases ("Canadian Cancer Society's Advisory Committee on Cancer Statistics,"). Cancer is also the most prevalent cause of pre-mature death in Canada, accounting for approximately 40% of the potential years of life lost (PYLL) which calculates death rates based on average life expectancy. It is estimated that on average, around 40% all Canadians will be diagnosed with cancer and a quarter of them will die from the disease. In Canada and the United States, the most common types of cancer include breast, lung, colorectal and prostate cancers ("Canadian Cancer Society's Advisory Committee on Cancer Statistics," ; Chaffer et al., 2013; "National Cancer Institute - Cancer Stastics,"). Lung cancer specifically accounted for an estimated 14% of new cancer diagnosis and 27% of all death from cancer in 2015, in Canada ("Canadian Cancer Society's Advisory Committee on Cancer Statistics,").

2.2 Cancer Metastasis

Tumors are solid- or fluid-filled masses of tissue. They can be either benign or malignant (cancerous). Most cancers can be broadly classified as either primary or metastatic (secondary). Primary cancers have tumors that are formed close to where the first cells started to divide uncontrollably. Although primary cancers can be fatal, the majority of cancer-related death are caused by primary cancers that have metastasized elsewhere (Mehlen & Puisieux, 2006). Metastatic cancers, are malignant tumors that form when invasive cells from the primary tumor leave their original site, and travel throughout the body via the vasculature and/or the lymphatic system before they settle in a site that is suitable for their survival (Hunter, Crawford, & Alsarraj, 2008).

The basics of cancer metastasis are similar in many cancers and depends heavily on the interaction of cancer cells with surrounding cells such as epithelial cells, endothelial cells immune cells and platelets (Yan & Jurasz, 2016). The first step of metastasis requires the dissemination of cancer cells from the primary cancerous tumor. This is a complex process, and in the case of carcinomas, cells that are stationary and epithelial in nature becomes invasive and gain motility in a process known as epithelial mesenchymal transition (EMT) (Son & Moon, 2010). Interestingly, a subset of sarcomas has been found to undergo the reverse process to EMT, known as mesenchymal epithelial transition (MET) (Somarelli et al., 2016). Genes such as those that codes for ZEB1 are involved in both EMT in carcinomas and MET in sarcomas (Chaffer et al., 2013; Somarelli et al., 2016). The process of EMT is physiologically important in a number of developmental processes including heart-valve formation and tissue and muscle formation

(Thiery, 2003). However, during cancer, EMT allows otherwise stationary cancer cells that are epithelial in origin to loose polarity and gain invasive phenotypes by switching out stable tight junctions for instable and leaky connections (Thiery, 2003, 2009). Next, these now invasive cells detach from the basement membrane and move towards either the blood vessel or the lymphatic system. Then, they would need to move through the endothelium layer covering the vessels and intravasate into the blood stream and/or the lymphatic system. This step depends heavily on enzymes such as MMPs and uPA that are able to degrade the basement membrane and extracellular matrix (ECM) (Radisky & Radisky, 2010). The second step of metastasis involves the cancer cell travelling within the circulation while surviving a number of factors such as shear force in the vasculature and immune cell surveillance. In this step, platelets play an important role in the survival of cancel cells within the vasculature by protecting them from shear stress and immune surveillance (Jurasz et al., 2004). The last step is where the metastatic cell arrest at the secondary site and extravasate into surrounding tissue. However, formation of metastatic tumors is also possible within the microvasculature where they have come to arrest (Weiss, Orr Fw Fau - Honn, & Honn, 1988; Wong et al., 2002). In order for cells with invasive phenotype to attach at their new environments, it is thought that the reverse of EMT, mesenchymal epithelial transition (MET), must happen. During MET, metastatic cells regain adhesion proteins such as E-cadherin and cell polarity, among other characteristics of epithelial cells (Yao, Dai, & Peng, 2011). However, still very little is known about the signalling mechanism behind the process of MET.

There are multiple theories of how a metastatic cancer cell can arise. Currently, the most accepted theory is the progression theory, originally proposed by Peter Nowell in 1976 (Nowell,

1976). This theory is based on the idea that cancers arise from a single cell. When a previously normal cell gains enough mutations for it to obtain certain proliferative advantages, it grows and divides continuously to form a colony of cells. The size of the colony is limited by the death of neoplastic cells by limiting nutrients and immune cells, until further mutations acquired by a single or a few cells within the colony give them further proliferative and survival advantages over other cells. These cells are allowed to proliferate, until more mutations gives rise to cells with more proliferative advantages. This sequential selection is what give rise to the small population of cells with metastatic ability (Nowell, 1976). Different metastatic potential of cells found within the clonal populations derived from a single parent cell line offers evidence in support for this theory (Fidler & Kripke, 1977). Additionally, there are distinct genetic predispositions within some cancer cells that not only affect their metastatic potential, but also the location of their metastasis. For example, subpopulations of breast cancer cells have genetic mutations that allow them to preferentially metastasize to the bone and lungs (Kang et al., 2003; Minn et al., 2005). There are also several tumor suppressor genes, such as genes coding for CD44 and MAPK4, whose expression can inhibit the formation of metastatic colonies by cancer cell lines without affecting primary tumor growth (Kauffman, Robinson, Stadler, Sokoloff, & Rinker-Schaeffer, 2003). This suggest that these genes may have regulatory effects on cancer metastasis and cells expressing these genes may be less likely to metastasize. However, newer theories such as the early oncogenesis model bring contradicting ideas. Most significantly, the early oncogenesis model contradicts the progression theory in that this theory suggest certain types of tumors have generally more invasive cells then other tumors. This idea arose from the observation that a large portion of cancer cells within tumors that are prone to metastasis express genetic signatures that are consistent with those of metastatic cells (Drukker et al., 2014;

Foukakis et al., 2015; van 't Veer et al., 2002). Based on these observations, the theory of oncogenesis suggest that the metastatic potential of a tumor is established in the early stages of normal cell mutations. This theory offers an explanation for patients with secondary tumors that have unknown primary origin, or very small primary tumors (Bernards & Weinberg, 2002).

It is thought that the location where metastatic cells arrest and extravasate are not random. Certain organs such as the lung, liver and bone tend to have higher chances of metastatic tumor formation. Since metastatic cancer cells travel via the vasculature as well as the lymphatic system, organs that are rich in microvascular structure and capillary beds are more like to have secondary tumor developments (Valastyan & Weinberg, 2011). Cancer cells, which are usually larger then red blood cells, are also more likely to be caught in the capillary beds due to their size (Chambers, Groom, & MacDonald, 2002). Moreover, recent advancement in the study of the role of the lymphatic system in tumor metastasis suggest that the lymphatic system aids in metastasis by generating more lymphatic vessels within the tumor in a process known as lymphangiogenesis, in addition to increase lymphatic flow (Harrell, Iritani, & Ruddell, 2007). However, the fact that certain types of cancer tend to metastasize to certain organs could not be explained simply by vasculature anatomy (Chambers, MacDonald, Schmidt, Morris, & Groom, 2000). For example, prostate cancer are most likely to metastasize to the bone, while ovarian cancers tend to metastasize to the abdomen (Hess et al., 2006). The observation that the location of cancer metastasis is not random lead to the proposal of the “seed and soil” hypothesis in 1889. The original theory suggested that circulating cancer cells had specific preferences for the location where the secondary tumors are formed, and that they will only grow when they have reached that destination (Paget, 1989). This theory was later refined by Fidler in 2003, whom

suggested that both the anatomy of the circulatory route as well as tumor microenvironment characteristic are important for the metastases (Fidler, 2003; Langley & Fidler, 2011). Microenvironment characteristic can influence the formation of secondary metastasis via the expression of proteins and receptors that allow preferential adhesion to target organ cells and the production of proteins that give survival advantages to the invading cancer cells. For example, bone metastases of breast cancers often overexpress parathyroid hormone related peptide (PTHrP), a protein that stimulate bone resorption. This resorption can stimulate the production of TGFβ, a protein highly expressed in many types of cancer and thought to promote cancer progression, from the bone matrix (Powell et al., 1991). Moreover, the lymphatic vessels can promote recruitment of cancer cells to the lymphatic system via the expression of chemotactic homing signals. SDF-1α was found to be expressed by the endothelial cells surrounding tumor-associated lymphatics. The high concentrations of SDF-1α then binds its receptor CXCR-4 expressed on cancer cells and promote invasion into the lymphatic systems (Hirakawa et al., 2009).

2.3 Cancer Stem Cells and the Origin of Cancer

The origin of cancer has long been an area of interest in research. It has been established that cancers are clonal in origin and arise from a single cell that has acquired single or multiple mutations (Greaves & Maley, 2012; Tomlinson, Sasieni, & Bodmer, 2002). The normal cell that obtain mutations which allow it to initiate cancer is known as the cell-of-origin. Additionally, mutations of different cells within the lineage hierarchy give rise to different subtypes of cancers within the same tissue (Visvader, 2011). There are several theories regarding how the first cells obtained mutations to become cancerous. The "Two-Hit Hypothesis", also known as the Kunson hypothesis, suggest that a cell needs two mutations to become tumorigenic. According to this theory, inherited mutations can make a person predisposed to have cancer. However, another acquired mutation is necessary for cells to become cancerous. Cells can also become cancerous if their mutations are retained later in life, again, two hits or two mutations are required (Knudson, 1971; Nordling, 1953). Another theory known as "The Mutator Phenotype Hypothesis" suggest that cancer arise from mutations in the genome that affects the integrity of DNA replication and repair. Thus errors in one round of DNA replication can be exponentially increased in the next round and so on (Loeb, Springgate, & Battula, 1974). However, these theories of cancer origin do not distinguish the difference between non-stem cell cell-of-origin and cancer stem cells (CSCs).

Although characteristics of CSC follow closely to that of the cell-of-origin, CSCs are not the same as cell-of-origin. CSCs may arise from the cell-of-origin or from downstream colonies that originated from the original colony (Visvader & Lindeman, 2008). It has been proposed that

characteristics of the cell-of-origin themselves, the type of mutation they acquire and environmental factors are what determines whether a particular cancer will take on the CSC phenotype (Visvader, 2011). This idea partially explain discrepancies reported by different researchers surrounding the existence of CSC in solid tumors in that perhaps some types of cancer simply do not sustain the types of mutations required for the formation of CSCs. Most criticisms of the CSC theory are focused on three aspects, namely the use of CSC markers, the use of xenotransplantation in animals to detect tumor initiating abilities and the lack of self-renewal abilities in some CSC populations. The identification of CSC markers allowed a much simpler method for the identification of CSC populations (Medema, 2013). However, recently the ability of current CSC markers to identify true CSCs is being brought into question. One laboratory reported that the expression of the CSC marker CD133 was not limited to tumor-initiating cells (Shmelkov et al., 2008; J. Wang et al., 2008). Moreover, the expression of CD133 can vary significantly between tumors types and even within a specific cell line. For example, in patients with glioblastoma, CD133 mRNA can vary between zero to up to twenty fold greater when compared to normal brain cells (Sakariassen, Immervoll, & Chekenya, 2007). Similarly, reported percentage of CSCs in the A549 human lung carcinoma cell line as identified by CD133 expression can vary between 0.2% to 20% (Bertolini et al., 2009). Additionally, it has been reported that difference in CSC identification techniques (e.g. FACS vs. immunohistochemistry) yielded varying results, which could also explain the variation in CSC percentage in cancer cell lines (Sakariassen et al., 2007). Although CSC markers are used widely in the identification of CSCs within a cell line, the self-renew and tumorigenesis ability of a given population of cells still needs verification by xenotransplantation into recipient animals. Critics of this method argue that this process preferentially selects cells that are more robust and invasive due to the extensive

processes of cell isolation, and thus underestimate the number of cells able to generate tumors (Kelly, Dakic, Adams, Nutt, & Strasser, 2007). Additionally, others have suggested CSCs are in fact regular cancer cells that have undergone EMT (Gupta, Chaffer, & Weinberg, 2009). Although techniques used to study CSCs may need re-evaluation or improvement, current evidence still provides a compelling argument in favor of the existence of CSCs or CSC-like cells and a role for these cells in metastasis (Medema, 2013; Reya, Morrison, Clarke, & Weissman, 2001; Visvader & Lindeman, 2008; F. Yang, Xu, Tang, & Guan, 2016).

CSCs are a small population of cancer cells with stem cell like characteristics, and first version of the cancer stem cell theory was proposed in 1867. It suggests that cancer arise form tissue specific stem or stem cell-like cells (Cohnheim, 1867). In this sense, the theories of cancer origin described above mostly pertained to the rise of the cell-of-origin, since they do not require the first cell to be stem cells. Although first proposed in 1867, evidence for the existence of CSCs did not appear until 1994 when subpopulations of tumor forming human acute myeloid leukemia (AML) cells were found. Only a small subset of AML cells with the CD34+CD38- phenotype was able to form new tumors when transplanted into immunodeficient mice, while other phenotypical subsets could not (Lapidot et al., 1994). Later, CSCs were identified in various solid tumors based on the expression or overexpression of CSC markers such as CD24, CD44, CD133, aldehyde dehydrogenase 1 (ALDH-1), Hoechst 33342-negative SP and ATP-binding cassette B5 (ABCB5) (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Ginestier et al., 2007; O'Brien, Pollett, Gallinger, & Dick, 2007; Schatton et al., 2008; C. Wu et al., 2007) (Table 3). The cancer stem cell theory also brings on important clinical implications. There is evidence that higher percentage of leukemic stem cells with the CD34+CD38-

phenotype is associated with poorer survival (van Rhenen et al., 2005). Similarly, evidence show higher percentage of cells with cancer stem cell phenotype are associated with higher chance of drug resistance, tumor regrowth, and poorer clinical outcomes in glioma, osteosarcoma, breast, colorectal, and lung adenocarcinomas (Cherciu, Barbalan, Pirici, Margaritescu, & Saftoiu, 2014; Ohi et al., 2011; Sholl, Barletta, Yeap, Chirieac, & Hornick, 2010; L. Wang, Park, Zhang, La Marca, & Lin, 2011; Zeppernick et al., 2008).

Table 3: Common cancer stem cell markers.

Cancer Type	CSC Marker	Reference
Lung	CD133, CD44, Hoechst 33342-negative SP	Roudi, Madjd, Ebrahimi, Samani, & Samadikuchaksaraei, 2014
Breast	ALDH-1	Ohi et al., 2011
Prostate	CD133	Shepherd et al., 2008
Glioma	CD133	Zeppernick et al., 2008
Brain	CD133	Singh et al., 2004
Ovarian	CD133, CXCR-4	Cioffi et al., 2015
Liver	CD90, CD133	Yang et al., 2008; Ma et al., 2007
Melanoma	CD133, Hoechst 33342-negative SP	Monzani et al., 2007

With the discovery of new evidence supporting the existence of CSC in both leukemia and solid tumors, the original cancer stem cell theory was revised to include the concept of tumor heterogeneity (Kreso & Dick, 2014). Tumor heterogeneity states that tumors arise from a single cell, and that all cells within a tumor are not identical (Park, Bergsagel, & McCulloch, 1971). The heterogenic nature of tumor masses means that within a given tumor, there are cells which vary in their invasiveness, phenotypes, metabolisms, functions, gene expressions and proliferative potentials (Lobo, Shimono, Qian, & Clarke, 2007). The current cancer stem cell theory proposes that only a small population of cancerous cells within a tumor have stem cell-like characteristics such as self-renew and the ability to produce differentiated cells with limited capacity to replicate. Thus, these cancer stem cells (CSCs) are responsible for initiating new tumors and produce progenitor cells, that in turn produce differentiated cells that makes up the tumor bulk. In this way, CSCs are able to maintain tumors while sustaining pools of progenitor cells (Kreso & Dick, 2014).

3. Platelet and Tumor Cell Interaction

3.1 Thrombosis in Cancer

Cancer patients are four times more likely to develop thrombosis compared to the rest of the population, and this risk may be elevated with some anti-cancer therapy (A. Y. Lee & Levine, 2003), and to date, thrombosis remains a major cause of death in cancer patients. The relationship between platelets and cancer was known since 1865 when the French physician Armand Trousseau correlated higher occurrences of thrombosis with cancer during the autopsies of patients whom died of cancer (Trousseau, 1865). Later on, it was discovered by that cancer patients have higher than normal platelet counts (Riess, 1872), and it was hypothesized that platelets form a thrombus around tumor cells and promote metastasis by providing protection in the vasculature and aid in adhesion of cancer cells to the endothelium prior to extravasation (Billroth, 1878). Further research showed that a decrease in experimental metastasis is associated with the inhibition of platelet function and a reduction of platelet count. For example, injecting neuraminidase in mice decreased metastasis by almost half because the injection produced thrombocytopenia in the host (G. Gasic & Gasic, 1962; G. J. Gasic, Gasic, & Stewart, 1968). This decrease in metastasis was confirmed by antiplatelet serum, other anti-platelet agents such as the P2Y12 inhibitor Ticagrelor, and in mice that have decreased platelet counts or have functionally deficient platelets (Camerer et al., 2004; G. J. Gasic et al., 1968; Gebremeskel, LeVatte, Liwski, Johnston, & Bezuhly, 2015).

3.2 Tumor Cell-Induced Platelet Aggregation

The reason cancer patients have a higher risk of thrombosis may be due in part to their more activated hemostatic system (Trousseau, 1865). Importantly, though a process known as tumor cell induced platelet aggregation (TCIPA), not only do cancer cells have the ability to activate platelets, activated platelets can in turn support the growth and metastasis of the tumor/cancer cells (Donati & Lorenzet, 2012; Jurasz et al., 2004). TCIPA is a complicated process governed by many different factors. For example, colorectal carcinoma cells were shown to express high levels of thromboxane synthase and thus generate high levels of TXA_2, a known platelet agonist (Sakai et al., 2006). Tumor cell lines can also activate platelets through the generation of potent platelet activators like thrombin and ADP (Zucchella et al., 1989). Moreover, the process of TCIPA was found to be MMP-2 dependent, and that the expression of MT-MMP-1 on platelet surfaces contributed to TCIPA via the activation of pro-MMP-2 on cancer cell surfaces (Alonso-Escolano, Strongin, Chung, Deryugina, & Radomski, 2004; Jurasz et al., 2001). More importantly, platelets activated by tumor cells release many platelet agonists and growth factors, including TXA_2, MMP-2 and VEGF, that contribute to further platelet and tumor cell activation (Menter et al., 2014).

TCIPA offers several physical advantages to the spread and growth of tumor cells through either direct (e.g. physical contact between platelets and cancer cells) or indirect (e.g. paracrine signalling) interaction between platelets and cancer cells. Platelet-tumor cell aggregates are able to protect tumor cells from sheer stress and immune cells within the vasculature by forming a protective shell, thus preventing clearance of the cell from circulation

during metastasis (Jurasz et al., 2004). Platelets are also able to secrete and thus provide many pro-growth and pro-angiogenic factors such as VEGF, PDGF, TGFβ, and insulin-like growth factors (IGF). These, among others, are highly beneficial for the development of tumors in that these proteins can induce immuno-suppression, angiogenesis and provide a preferential environment for tumor proliferation (Goubran, Stakiw, Radosevic, & Burnouf, 2014; Hara, Steiner, & Baldini, 1980). In addition to stimulating tumor growth, these factors as well as others released during TCIPA including SDF-1α and MMPs, are also important in promoting cancer cell metastasis (Shen et al., 2009). The thrombi formed around tumor cells aid in extravasation by either sticking to the endothelium or blocking microvessels (Rickles, 2001). Furthermore, platelets are considered to be a part of the tumor's microenvironment, since tumors require access to blood to grow. Tumor microenvironment consists of the tumor cells themselves, non-malignant cells, and connective tissue cells that contribute to the structure and survival of the tumor. Importantly, platelets are not only a part of the microenvironment, but they also interact with other cells in the tumor microenvironment, such as endothelial, epithelial and immune cells (Yan & Jurasz, 2016). By stimulating processes such as EMT, endothelial barrier permeability and inhibition of natural killer cells, platelets contribute greatly to making the tumor microenvironment favorable for metastasis (Amo et al., 2014; Labelle, Begum, & Hynes, 2011; Schumacher, Strilic, Sivaraj, Wettschureck, & Offermanns, 2013).

3.3 Platelet-Derived Factors Affecting Cancer Metastasis

As mentioned, not only do platelet offer direct physical protection to cancer cells during metastasis, factors released from platelets can also promote metastasis. Platelet-derived factors such as VEGF stimulate angiogenesis and the formation of leaky blood vessels within tumors, thus giving tumor cell access to the blood stream (Weidner, 2002). Furthermore, platelet-derived S1P and lysophosphatidic acid (LPA) contribute to the increase in endothelial permeability to aid cancer cell extravasation (Gay & Felding-Habermann, 2011). Another platelet-derived factor that was shown to affect metastasis is SDF-1α. SDF-1α is a chemokine found to be important in the mobilization of cancer cells, but is also a potent platelet agonist (Chatterjee & Gawaz, 2013). SDF-1α has been implicated in stimulating the proliferation and metastasis of ovarian, pancreatic, breast and lung cancers among others (Brennecke et al., 2013; Q. Guo et al., 2014; Luker et al., 2012; Shakir et al., 2015).

SDF-1α, or stromal derived factor-1α is a chemokine that is encoded by the human CXCL12 gene. There are two classes of chemokines, defined by the location of conserved cysteine residues within the protein, and SDF-1α belongs to the C-X-C class where the cysteine residues are separated by amino acid residues (Graves & Jiang, 1995). SDF-1α elicits its effects via interaction with its receptor C-X-C chemokine receptor 4 (CXCR-4), and to an extent, chemokine receptor 7 (CXCR-7) (Bleul et al., 1996; Burns et al., 2006). SDF-1α is a potent chemotactic protein physiologically involved in the recruitment cells such as bone marrow derived stem cells, progenitor cells, endothelial progenitor cells and smooth muscle cells during embryogenesis and wound healing (Massberg et al., 2006; Nemenoff et al., 2008; Petit et al.,

2002; Stellos & Gawaz, 2007; Zernecke et al., 2005). Additionally, it was found that SDF-1α is able to induce endothelial progenitor cell differentiation from human CD34+ cells (Stellos, Langer, et al., 2008). SDF-1α is also a strong chemoattractant for lymphocytes as well as a inducer of macrophage differentiation, suggesting its involvement in immune reactions (D. K. Jin et al., 2006; Sanchez-Martin et al., 2011). Moreover, SDF-1α was found to have the ability to induce CD4+ T-cell apoptosis, and as a result regulate their numbers by increasing T-cell Fas ligand expression (Colamussi et al., 2001). Although SDF-1α is secreted by platelets, a significant proportion of it remains bounded to the platelet surface (Chatterjee et al., 2011). Since the membrane bound SDF-1α can stimulate mobilization of progenitor cells to the vasculature, it could be reasonable to speculate that they, as well as other factors that affect stem cell signalling will have similar mobilizing effects on cancer cells and CSCs (Stellos et al., 2009) (Figure 4). Moreover, SDF-1α can also contribute to metastasis through the activation of platelets via PKC and downstream extracellular signal-regulated kinase (ERK) and Akt pathways (Karim et al., 2016).

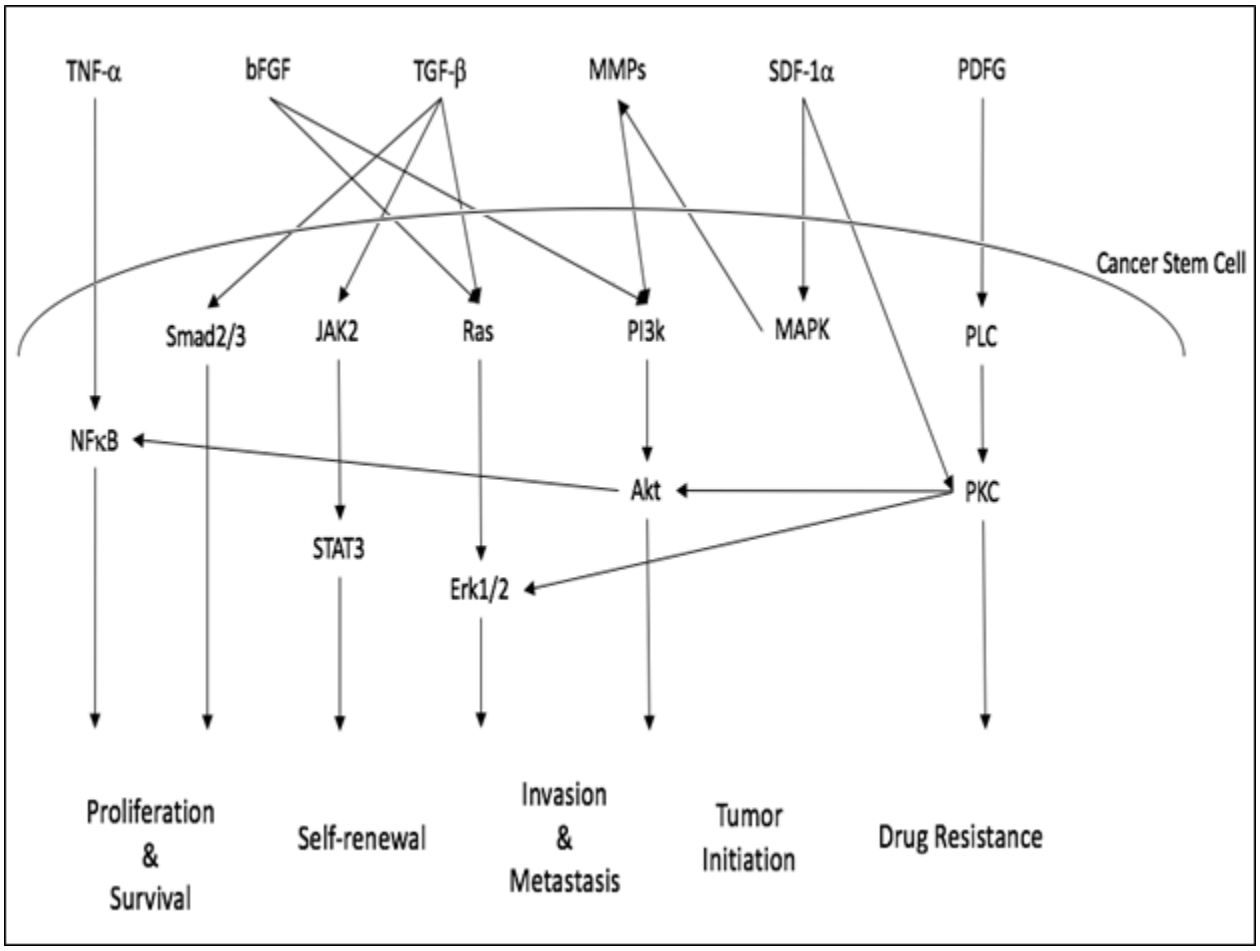


Figure 4: Platelet-derived factors potentially affecting cancer stem cell signalling.

SDF-1α induces cell motility partly through the up-regulation of matrix metalloproteinases (MMPs) dependent manner, which are responsible for degrading the ECM to facilitate cancer cell invasion (Tang, Tan, Fu, & Yang, 2008). The interaction between SDF-1α and CXCR-4 leads to an increase in the expression of MMP-2 and MMP-9 in cancer cells (Pan et al., 2013; Tang et al., 2008). This increase was found to be associated with stem and cancer stem cell trafficking (Massberg et al., 2006). Moreover, the MMPs were also found to promote tumor metastasis to preferred niches through inducing tumor cell dissemination and increasing vascular permeability (Farina & Mackay, 2014). For example, the SDF-1α signalling pathway was found to be important in the migration of breast cancer, glioblastomas and lung cancer cells, in an MMP dependent manner (Osman & Osman, 2016; Smith et al., 2004; Tseng, Vasquez-Medrano, & Brown, 2011).

MMPs are part of the metzincin superfamily of zinc-dependent peptidases, in which all members share a common zinc binding motif: HEX-XHX-XGX-XH (Bode et al., 1996). Most MMPs contain three common domains: the pro-domain that contains a cysteine switch motif PRCGXPD attached to a hinge region; a catalytic domain with the zinc binding motif with a zinc ion and up to three calcium ions that stabilize the structure; and a hemopexin domain. Currently, there are 24 identified MMPs in humans, all of them are either secreted or membrane bound. MMPs are commonly classified by either their preferred substrate or domain organization. All MMPs are synthesized in the pro-form and require activation (Verma & Hansch, 2007). The cysteine "switch" in the MMP pro-domain binds the zinc in the active site, thus inactivating the enzyme. The membrane bound MMPs as well as a few others, including MMP-11 and MMP-23, contain a furin activation sequence. These MMPs are activated intracellularly by furin, a serine protease in the trans-Golgi network (TGN) that traffics secretory proteins to their destinations,

and are thus secreted as active MMPs. Other MMPs are secreted as pro-MMPs and can be activated by the direct cleavage of the pro-domain by serine proteases such as plasmin, or other active MMPs including MMP-3, MMP-10 and membrane type MMPs. These MMPs can also activate though the autoproteolysis of their pro-domain as a result of conformation change and reduction of the free cysteine by reactive oxygen species (Nagase, 1997). Endogenous TIMPs have also been identified, and they regulate the expression and function of MMPs. TIMPs inhibit MMPs by chelating the functional zinc in the MMP active site. There are four known TIMPs, each with different specificity for different MMPs. For example, while TIMP-1 is only a potent inhibitor for MMP-3 and -7, TIMP-2 inhibits all MMPs. The physiological and pathological effects of MMPs are ultimately dependent on the balance between MMPs and TIMPs (Brew & Nagase, 2010). Physiologically, MMPs are known to be important for extracellular matrix (ECM) remodeling during embryogenesis, angiogenesis and tissue repair (Loffek, Schilling, & Franzke, 2011). MMPs has also been implicated under pathological conditions to influence cancer metastasis, atherosclerotic plaque rupture and ventricular remodeling after myocardial infraction (Phatharajaree, Phrommintikul, & Chattipakorn, 2007). However, recent development brought to light other functions of MMPs, including the cleavage of cell surface receptors, activation of cytokines, and the release of trapped growth factors and apoptotic proteins (Rodriguez, Morrison, & Overall, 2010).

Together, the SDF-1α: CXCR-4: MMP signalling pathway has been found to be important in the metastasis of a variety of cancers. SDF-1α and CXCR-4-mediated metastasis was found to be dependent on ERK-1/2 activation in ovarian and colorectal cancers (Brand et al., 2005; Shen et al., 2009). SDF-1α and CXCR-4-mediated metastasis in prostate cancer was found

to be dependent on the phosphorylation of Akt pathway, and downstream release of MMP-9 (Chinni et al., 2006). Moreover, MMP-9 dependent invasion of MCF7 human breast cancer cell line was dependent on the presence of platelets (Alonso-Escolano et al., 2006). These evidence highlight the importance of the SDF-1α: CXCR-4: MMP signalling pathway in cancer growth and metastasis. However, not much is known regarding the role of platelets, a major source of SDF-1α and MMPs in the vasculature, in SDF-α: CXCR-4 mediated metastasis.

Hypothesis & Objectives

1. Rationale

2. Hypothesis

3. Study Objectives

1. Rationale

Platelets are a known to facilitate the recruitment and mobilization of stem/progenitor cells (Sopova, Tatsidou, & Stellos, 2012; Stellos, Gnerlich, Kraemer, Lindemann, & Gawaz, 2008). Stem cells and cancer stem cells share many similarities such as the expression of various genes and receptors and proliferative characteristics, although those that govern cell proliferation, are significantly higher in CSCs (Ben-Porath et al., 2008; Santagata, Ligon, & Hornick, 2007; Sperger et al., 2003). For example, it was found that the mRNA expression patterns of more than 300 genes was higher in malignant stem cells of cancer patients than in normal human stem cells (Birnie et al., 2008). Moreover, like somatic stem cells, CSCs are able to self-renew by producing two daughters: one that is identical to the parent cell and another progenitor cell that produce more differentiated cells and have limited capacity to replicate (He, Nakada, & Morrison, 2009). Thus, it is reasonable to speculate that platelets, which mobilize normal stem cells may also mobilize CSCs (e.g. stimulate their invasion/ intravasation/ extravasation) in a similar manner. This has not been previously investigated and may be particularly pertinent to understand since platelets are known to facilitate metastasis and CSCs have been speculated to be the cells important in initiating and maintaining metastasis. If so, the platelet initiated signalling pathways, which mediate stem cell mobilization, may also be responsible for stimulating CSC invasion.

One such signalling pathway that is shared between stem cell mobilization and cancer cell metastasis is the SDF-1α: CXCR-4: MMP-2/9 signalling pathway (Lapidot & Kollet, 2002; Massberg et al., 2006; Smith et al., 2004). Many have demonstrated the importance of SDF-1α

and MMPs in vascular remodeling and angiogenesis, which are unfortunately also important for tumor growth and metastasis (F. Jin et al., 2008; Kollet et al., 2003; Petit, Jin, & Rafii, 2007). However, although there is evidence linking SDF-1α, MMP-2 and -9 with cancer metastasis, this pathway has not been studied in the context of platelet-CSC interaction.

Such a formative study may be simplified by studying platelet interactions with a well-documented cell line that is enriched with stem-like cancer cells. one such cell line is the human A549 lung carcinoma cell line (Ho, Ng, Lam, & Hung, 2007). Additionally, the A549 cell line has previously been well characterized in studies investigating the role of MMPs in platelet-cancer cell interactions (Janowska-Wieczorek et al., 2005; Jurasz et al., 2001); thus, making it an excellent cell line to work with on initial studies into platelet-CSC interactions.

2. Hypothesis

I hypothesize that platelet derived SDF-1α can preferentially stimulate the invasion of the cancer stem cells of the A549 human lung carcinoma cell line, and that this preferential stimulation is dependent on the up-regulation of MMPs in cancer stem cells.

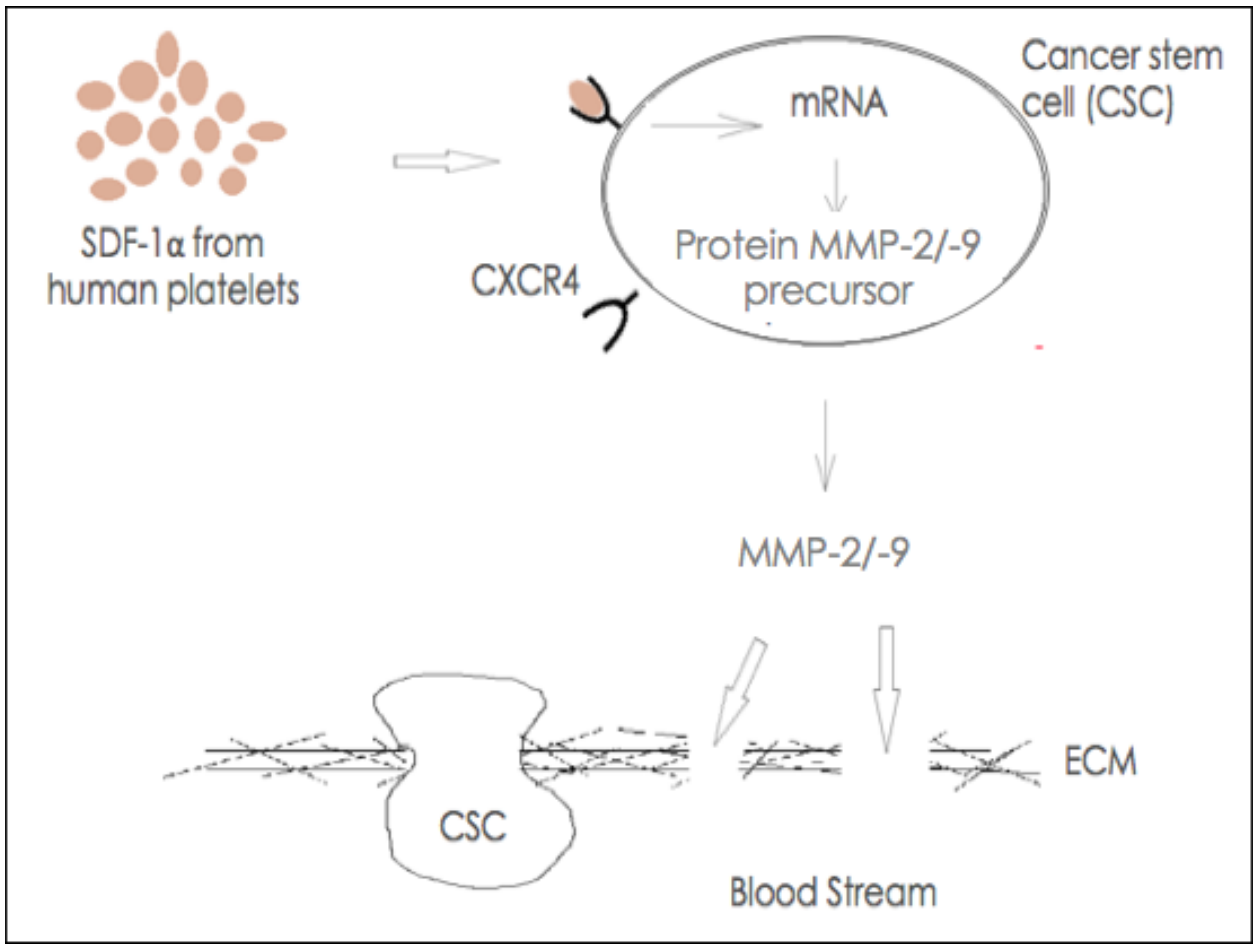


Figure 5: A schematic of the hypothesis. SDF-1α from platelets preferentially binds to its receptor CXCR-4 on cancer stem cell surfaces, leading to increased cancer stem cell invasion via increased MMP-9 production.

3. Study Objectives

The objectives of this study include:

1. To identify the cancer stem cell population in the A549 human lung carcinoma cell line.

2. To determine whether releasates from collagen-aggregated human platelets can preferentially stimulate A549 cancer stem cell invasion.

3. To investigate whether platelet can stimulate changes in MMP-2 and MMP-9 production by cancer and cancer stem cells of the A549 cell line.

4. To investigate whether platelet-induced A549 CSC invasion is dependent on SDF-1α and CXCR-4 interactions.

Materials & Methods

1. Reagents

2. Methods

1. Reagents

Gelatin was purchased from Chrono-log (Havertown, PA, USA). Hoechst 33342 was purchased from Life Technologies (Carlsbad, CA, USA) and anti-CD133-Phycoerythrin (PE) was from Miltenyi Biotec (Gladbach, Germany). Fc Receptor Saturating Reagent was obtained from Southern Biotech (Birmingham, AL, USA) and IgG2bκ isoform control conjugated to PE was from eBioscience (San Diego, CA, USA). AMD3100, GM6001, cell tracker dye CMFDA and cell tracker dye CMTPX was purchased from Sigma Aldrich (St. Louis, Mo, USA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

2. Methods

2.1 Cell Culture

A549 human lung carcinoma cell line cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and an antibiotic cocktail containing gentamycin (5mg/ml), penicillin (6mg/ml) and streptomycin (1mg/ml) and grown in a humidified cell incubator at 37°C with 5% carbon dioxide. Fresh culture medium was supplied every two days and cells were passaged as needed. Cells were passaged using Trypsin-Ethylenediaminetetraacetic acid (Trypsin-EDTA) solutions and gentle shaking to detach cells. Trypsin-EDTA was then neutralized with twice the amount (v/v) of culture medium after the cells detach and the cells were pelleted at 130 relative centrifugal force (RCF) for 7 minutes. Cells with passage number between 5 and 35 was used for all experiments.

2.2 Preparation of Washed Human Platelets

Following informed consent, 36ml of blood were obtained from healthy human donors between the ages of 18 and 50 whom had not taken any platelet affecting drugs within two weeks of the blood draw. Blood was drawn into 4ml of room temperature sodium citrate. Platelets were washed according to the prostacyclin-washed platelet protocol described by Radomski and Moncada (M. Radomski & Moncada, 1983). Briefly, 3µl of prostacyclin (0.06µg/ml) were added to the blood before centrifuging at 200 RCF for 20 minutes in an Eppendorf 5810R Centrifuge (Eppendorf, Hamburg, Germany). Platelet rich plasma (PRP) were obtained and moved to a clean 50ml tube and an additional 0.3µg of prostacyclin were added per milliliter of PRP. The PRP were then spun at 900 RCF for 10 minutes to pellet the platelets. Without re-suspending, the resulting platelet pellet was washed three times with room temperature Tyrode's buffer. The platelets were then re-suspended in Tyrode's buffer, counted using a hemocytometer (Assistant, Sondheim, Germany) and diluted with Tyrode's buffer to a concentration of 2.5×10^8 platelets/ml.

2.3 Platelet Aggregation & Platelet Releasate Collection

The aggregation of washed human platelets was performed in a Chrono-log Dual Channel Lumi-Aggregator (Model 560, Chrono-log, Havertown, PA, USA). Saline (10μl/ml) for resting platelet releasate, or collagen (10μg/ml) for activated platelet releasate, was added to the platelets after 2 minutes of incubation within the aggregometer to establish baseline light transmittance. The changes in light transmittance were recorded for a further 4 minutes using the AGGRO-LINK software (Chrono-log, Havertown, PA, USA). After aggregation, 1μg of prostacyclin was added to the resting platelet releasates to prevent aggregation during centrifugation, and all platelet samples were spun at 10,000g in a Sorvall Legend Micro 17 centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) to separate the platelets/platelet aggregates from releasates, and the releasates were then stored at -80°C until ready to use.

2.4 Measurement of SDF-1α levels in Platelet Releasates

Resting and collagen-aggregated platelet releasate were obtained as described above. Enzyme-linked immunosorbent assays (ELSIA) were performed using the Human CXCL-12/SDF-1 alpha Quantikine ELISA Kit (R & D Systems, Minneapolis, MN, USA) following the contained protocol. Briefly, 100µl of sample were added to each well in the ELISA plate and incubated for 2 hours at room temperature. Wells were washed with wash buffer provided with the kit and dried. 200µl of SDF-1α conjugate were added to each well and the plate was incubated for another 2 hours at room temperature. The plate was washed again at the end of the incubation period and 200µl of Substrate Solution from the kit was added. An incubation of 25 minutes in the dark was followed by the addition of 50µl Stop Solution from the kit. The plate was then analyzed using a iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, USA). Concentrations of SDF-1α were calculated based on a standard curve and expressed as pg/ml.

2.5 Identification of Cancer Stem Cell Based on Hoechst Negative Side Population Staining

Hoechst 33342-negative SP identification of CSC was based on the Goodell protocol (Goodell, 2005). Hoechst 33342 is a fluorescent nuclear dye that stains DNA. The SP is distinguished from the general cell population by their ability of efflux the Hoechst dye via the overexpression of the ATP-binding cassette sub-family G member 2 (ABCG2) protein (Goodell, Brose, Paradis, Conner, & Mulligan, 1996). The assay was originally established for the identification of hemapoietic stem cells. However, since CSCs expresses many of the characteristics of regular stem cells, this protocol has also been extensively used for the identification of CSCs (Hirschmann-Jax et al., 2004). Hoechst 33342 excites at 350nm and has its maximum emission at 461nm. Often, Hoechst is excited with an ultraviolet lamp. The emission is measured in the FL1 channels (450/50 nm band-pass filter), which detects the blue Hoechst fluorescence. However, due to the brightness of the Hoechst signals, the FL3 channels (675/20 nm long-pass filter) can also detect some red Hoechst fluorescence. In order to obtain a better distinction of the SP, the red and blue fluorescence are plotted against each other (e.g. FL3 vs. FL1) (Goodell et al., 1996).

A549 human lung carcinomas were trypsinized, washed and re-suspended in 1ml DMEM + 2% FBS at a concentration of ~1 x 10^6 cells/ml. Hoechst 33342 were added at a final concentration of 5µg/ml and incubated at 37°C in the dark for 1.5 hours with occasional shaking. After incubation, cells were washed and re-suspended with 1ml ice cold PBS + 2% FBS before

analysis using a Cell Lab Quanta SC MPL Flow Cytometer (Beckman Coulter INC., Fullerton, CA, USA). 10,000 events were collected for all samples.

2.6 Identification and Isolation of Cancer Stem Cell Based on CD133 Staining

Cells from the A549 human lung carcinoma cell line were detached from the culture flasks using 7mM Ethylenediaminetetraacetic acid (EDTA) in DMEM with 10% FBS and pelleted at 130 RCF for 7 minutes. The resulting pellet was washed and re-suspended with CD133 Staining Buffer containing PBS with 2mM EDTA and 0.3% FBS. Fc Receptor Saturating Solution and either mouse anti-human CD133/2- phycoerythrin (PE) antibody or mouse IgG2bκ-PE antibody (eBioscience, San Diego, CA, USA), as an IgG isotype control, were added to the cells and incubated for 10 minutes at 4°C in the dark. The cells were then pelleted at 5,000g for 5 minutes, washed and re-suspended in fresh CD133 Staining Buffer for analysis using a Cell Lab Quanta SC MPL Flow Cytometer (Beckman Coulter INC., Fullerton, CA, USA).

To separate A549 cells into CD133-positive and –negative subpopulations, A549 cells were prepared as described above and kept on ice until ready for sorting using a BD FACSAria III Fluorescence Activated Cell Sorter (FACS) (BD BioSciences, Mississauga, ON, Canada). Sorted A549 cells were pelleted at 130 RCF for 7 minutes and an equal number of either population (CD133-positive and CD133-negative) was re-suspended into 1ml of DMEM warmed to 37°C. The cells were then either seeded into 6-well plates for zymography or stained with cell tracker dyes for invasion assay. The exact number of cells used for each experiment depended on the yield of the particular sort and was between 10,000 and 70,000 cells per population.

2.7 Invasion Assay Using Modified Boyden Chamber

A549 cells were cultured and detached from flasks as described above. A total of 2 x 10^6 cells were re-suspended in DMEM without FBS and added to each gelatin (1mg/ml)-coated 6 well plate-inserts (PET, 8.0μm, Corning, NY, USA) and placed in 6 well plates. Gelatin-coated inserts were used to mimic the ECM found in physiological conditions. 1.5ml of either resting or collagen-aggregated platelet releasates were added to the bottom of the inserts and the system was incubated in a humidified cell incubator at 37°C with 5% carbon dioxide. After 24 hours, the cells at the bottom of the insert were detached using trypsin-EDTA and re-suspended in DMEM warmed to 37°C. The cells were then stained with Hoechst-33342 and analyzed using a Cell Lab Quanta SC MPL Flow Cytometer (Beckman Coulter INC., Fullerton, CA, USA). The percentage of invaded Hoechst-negative SP vs. non-SP A549 cells were measured as described above.

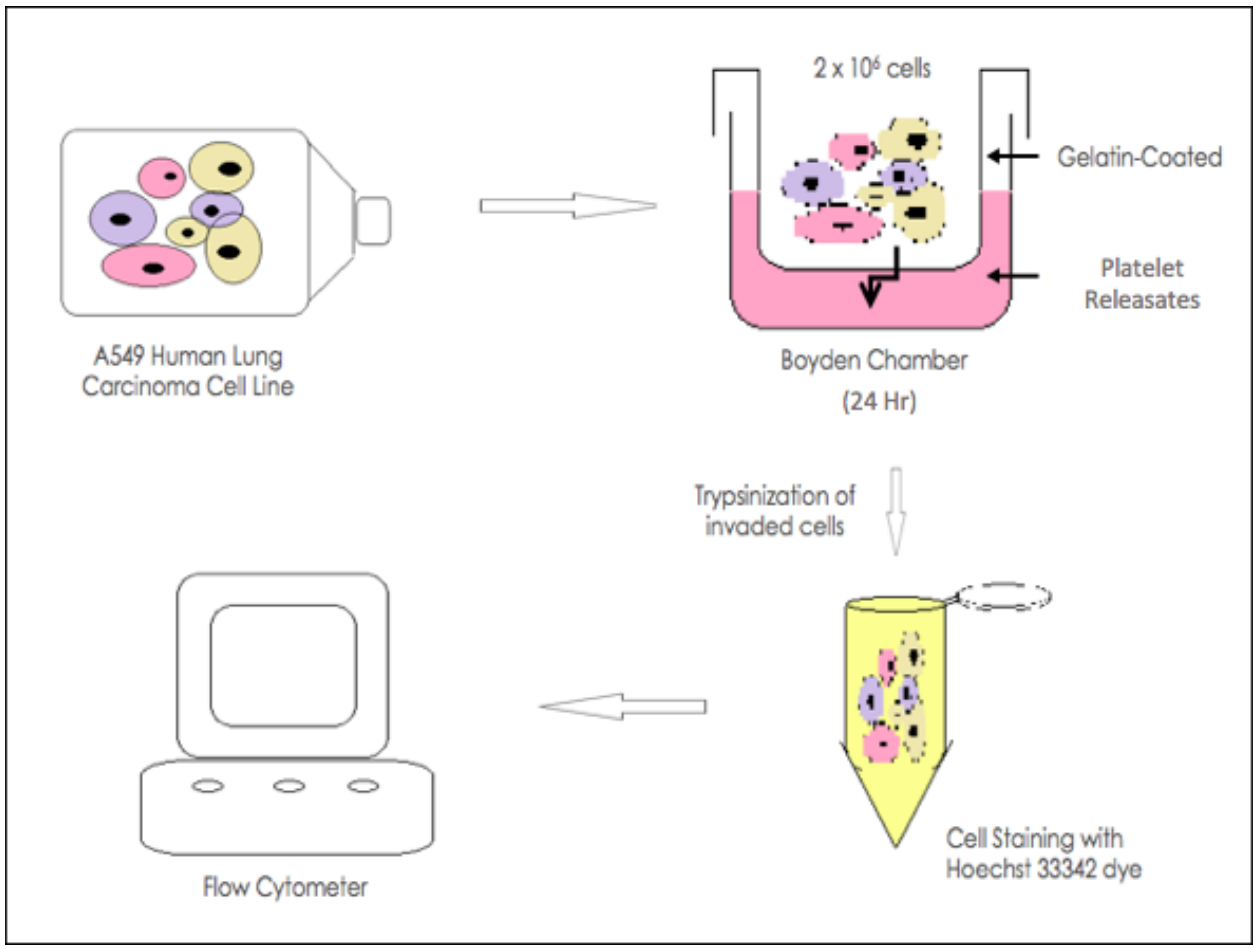


Figure 6: A schematic of SP-identified CSC invasion assay. Gelatin (1mg/ml) was added to the Boyden chamber and incubated at 37°C for 1 hour, and then air dried for 30 minutes. 2,000,000 A549 cells re-suspended in DMEM without FBS were placed in the filter and platelet releasates that were collected after 6 min of aggregation were added to the bottom of the chamber. The entire system were incubated at 37°C for 24 hours. Following invasion, the cells that have invaded were removed from the bottom of the chamber using trypsin and were stained with Hoechst 33342 (5µg/ml), and analyzed using the flow cytometer.

For invasion assays in which A549 CSCs and non-CSCs were identified based on CD133, A549 cells were sorted based on the presence or absence of the CD133 surface marker using a BD FACSAria III flow cytometer (BD BioSciences, Mississauga, ON, Canada) as described above. Equal numbers of CD133-positive and CD133-negative cells were re-suspended in DMEM without FBS. Again, the number of cells for each specific experiment depended on the yield of individual cell sorts. Approximate range of cells used were between 10,000 and 70,000 cells per population. The cell tracker CMFDA (5μM) was added to CD133-positive cells and CMTPX (5μM) was added to the CD133-negative CD133 cells and both were incubated at 37°C for 45 minutes in the dark. After staining, the CD133-positive and negative populations were combined, re-suspended in 500μl DMEM without FBS warmed to 37°C and added to gelatin (1mg/ml)-coated 24 well plate-inserts (PET, 8.0μm, Corning, NY, USA) placed in a 24 well plate. 0.5ml of either resting or collagen-aggregated platelet releasates were added to the bottom of the inserts and incubated in a humidified cell incubator at 37°C with 5% carbon dioxide. After 24 hours, the cells on top of the insert were removed by scraping with cotton swabs and the insert was cutted out and placed on a microscope slide. A coverslip was placed on top of the insert and sealed with clear nail polish. Inserts were viewed with an Olympus IX-81 WAVEFX spinning disk confocal microscope (Quorum Technologies, Guelph, ON, Canada) using the 10x dry objective at room temperature. LMM5 50mW 491nm pumped diode laser and 50mW 561nm pumped diode laser (Spectral Applied Research Inc., Richmond Hill, ON, Canada) were used to illuminate the samples. The insert cutout was superimposed with a 3 x 3 grid and one image was acquired from each area of the grid for a total of nine images (field of view) per insert with the EM-CCD (C9100-13) camera (Hamamatsu Photonics, Shizuoka, Japan) connected to the Velocity software (Perkin Elmer Inc., Waltham, MA, USA). The number of cells invaded per

field of view was counted and the average number of invaded cells were determined and expressed as percent per field of view (Figure 7).

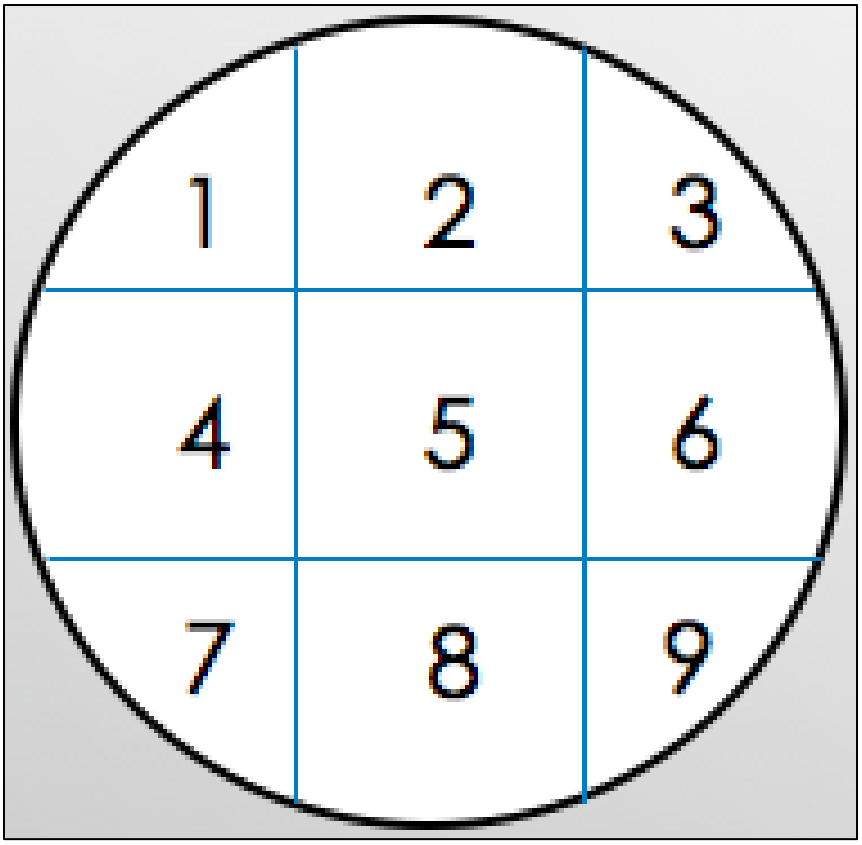


Figure 7: Superimposed grid on Boyden chamber inserts for invasion assay. One image is taken from a field of view in each area of the grid. A total of nine images were taken per insert

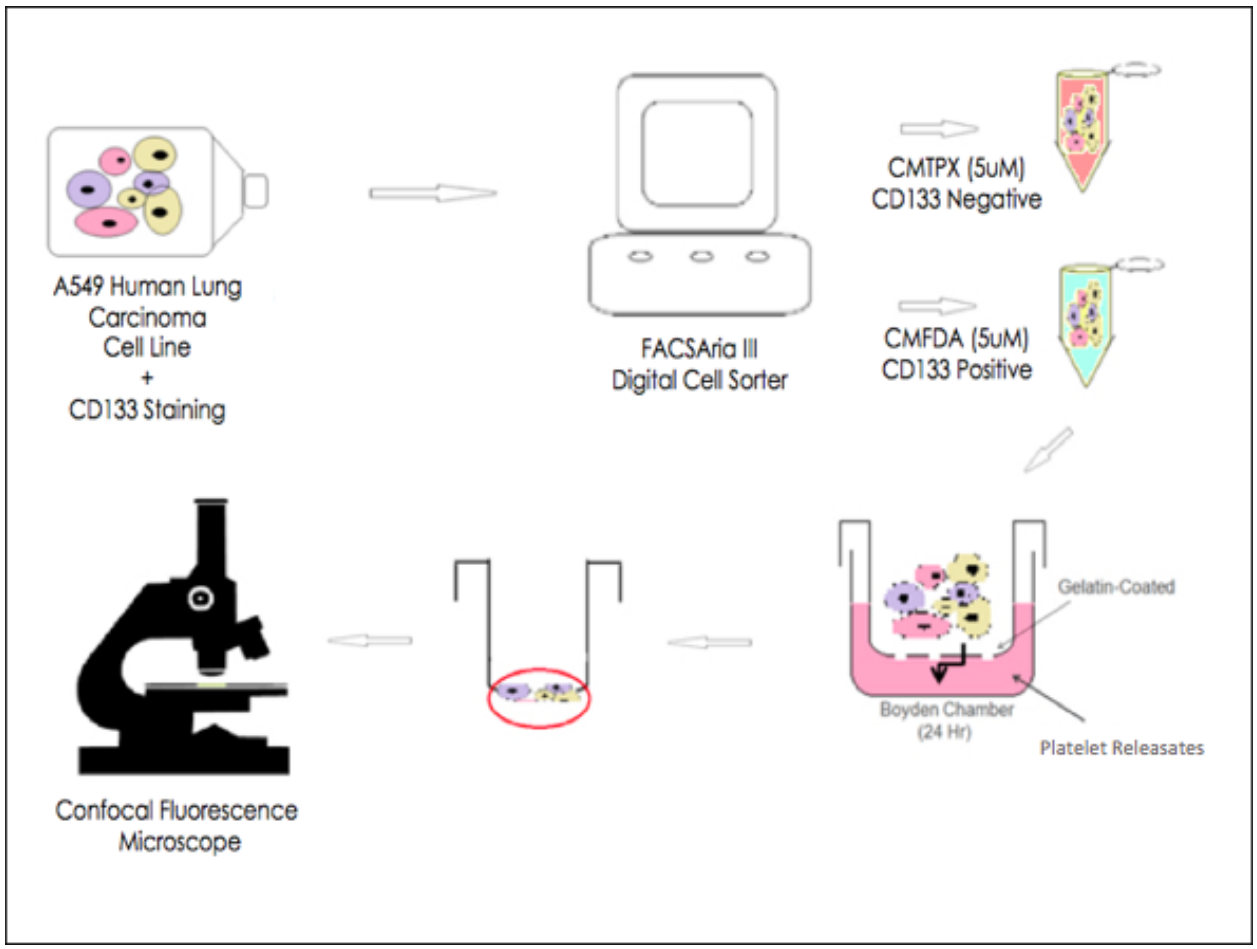


Figure 8: A schematic of CD133-identified CSC invasion assay. Gelatin (1mg/ml) was added to the Boyden chamber and incubated at 37°C for 1 hour, and then air dried for 30 minutes. Same numbers of CD133-positive and CD133-negative A549 cells were stained with either CMFDA (5μM) or CMTPX (5μM) for 45 minutes at 37°C in the dark. The populations were then combined and placed in the Boyden chamber with collagen-aggregated platelet releasates collected after 6 minutes of aggregation for 24 hours. After the incubation, the membrane with the cells were removed and viewed under an epifluorescence confocal microscope.

For invasion assays which were performed in the presence of either the CXCR-4 inhibitor AMD3100 (10μM) or the MMP inhibitor GM6001 (10μM). Before the inhibitors were used in the invasion assay, Annexin-V staining was used to test the toxicity of these inhibitors on A549 cells. A549 cells were cultured as described above and were incubated with either DMEM (no treatment control), AMD3100 (10μM in DMEM), Phenanthroline (1mM) or GM6001 (10μM in DMEM) for 24 hours at 37°C before the experiments. Following treatment, cells were detached from flasks as described above and pelleted at 130 RCF for 7 minutes. Detached cells were re-suspended in binding buffer (0.1M HEPES, 1.4M NaCl, 25mM $CaCl_2$, pH 7.4) and incubated with Annexin V- fluorescein (FITC) for 15 minutes in the dark. The samples were then analyzed using a Cell Lab Quanta SC MPL Flow Cytometer (Beckman Coulter INC., Fullerton, CA, USA). Cells positive for Annexin V staining are considered as apoptotic.

For the invasion assays, the inhibitors AMD3100 (10μM) and GM6001 (10μM) were added to the top of the insert and activated platelet releasates were added to the corresponding wells of the inserts. The inserts were then analyzed based on either Hoechst 33342 or CD133-staining as described above to identify the CSC and non-stem cancer cells.

2.8 Sample Preparation for the Measurement of A549 MMP-2 & -9 Levels

Following FACS, equal numbers of CS133-positive and CD133-negative A549 cells (between 10 x 10^3 and 20 x 10^3 cells for each fraction) were re-suspended in DMEM with 10% FBS and seeded into 24 well plates. When the cells became confluent (3-5 days), 1ml of either freshly prepared prostacyclin-washed platelets or Tyrode's buffer (control) were added to the cells, and incubated for 24 hours at 37°C. The cells were detached from the plates using trypsin-EDTA solution the following day and pelleted at 130 RCF for 7 minutes using the Eppendorf 5810R Centrifuge (Eppendorf, Hamburg, Germany). The resulting pellet was frozen at -80°C until ready to use.

2.9 Measurement of A549 cell MMP-2 & -9 Levels Using Gelatin Zymography

Zymography was performed using 8% sodium dodecyl sulfate-polyacrylamide gel with gelatin (2mg/ml) as substrate, according to a previously published protocol (Jurasz et al., 2001). Briefly, the sample pellets were re-suspended in homogenizing buffer. The samples were then sonicated on ice using the 1.5 setting on a Misonix XL2000 series sonicator (Misonix Inc., Farmingdale, NY, USA) for three times, 5 seconds each and centrifuged for 5 minute at 5,000g. 5µl of 4x loading buffer were added to 15µl of the resulting supernatants to make a total of 20µl of sample that were loaded per lane. A molecular weight protein standard was also loaded. The gelatin-incorporated sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel was run on ice at 150 volts until the running buffer dye ran out (~1 hour 45 minutes). After electrophoresis, the gels were cut at around the 50 kDa marker as indicated by the protein standard.

The top gels were washed three times for 20 minutes with 2% Triton X-100 followed by two 20 minute washes with zymography buffer containing Tris-HCl buffer with 0.15M NaCl, 5mM $CaCl_2$ and 0.05% NaN_3 at pH 7.6, at room temperature. Finally, the gels were incubated in zymography buffer at 37°C for 2-3 days. The gels were then stained with a 0.05% Coomassie Brilliant Blue solution for 2 hours and de-stained with a de-staining solution with 4% ethanol and 8% acetic acid.

The bottom gel was transferred using a semi-dry method onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, USA) and blocked in 5% skim milk in Tween-Tris

buffered saline (TTBS) wash buffer at 4°C overnight. The membranes were then incubated with rabbit anti-human β-actin-horse radish peroxidase (HRP) (1/40,000) for 30 minutes at room temperature and visualized with the Amersham ECL Prime Western Blotting Detection Reagent kit (GE Healthcare, Little Chalfont, UK).

The gelatinase activity of MMP-2 and MMP-9 was analyzed based on the densitometry of the bands using a Versa Doc 5000MP (Bio-Rad, Hercules, CA, USA), and expressed in arbitrary units of density normalized to β-actin.

2.10 Measurement of MMP-2 mRNA Levels Using Real Time PCR (RT-PCR)

mRNA of platelet pellets from FACS-sorted A549 cells treated with platelets were isolated using the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada). The mRNA was then reverse transcribed using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time PCR was performed using a SYBR Green PCR Master Mix (KAPA Biosystems, Wilmington, MA, USA) in an Applied Bioscience 7500 Real Time PCR System (Waltham, Massachusetts, USA). 10ng of cDNA were used for each qPCR reaction with 200nM of either MMP-2 (Forward: GAT GGA TAC CCC TTT GAC GGT; Reverse: GCT GTT GTA CTC CTT GCC ATT G) primer pairs. 200nM of ribosomal protein L-32 (RPL-32) (Forward: TGC CCA ACA TTG GTT ATG GA; Reverse: TGG GGT TGG TGA CTC TGA TG) primer pairs were used as an endogenous control. The amount of PCR product was calculated using the δδCT method and normalized to the house keeping gene RPL-32.

2.11 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software INC, La Jolla, CA, USA). One-way analysis of variance (ANOVA) with either Tukey's or Dunnett's post-test was used to analyze variance between experiments with more than two groups of data where appropriate. For comparison between two groups of data, paired student's t-tests were performed. N number reflects the number of independent experiments performed. All data are reported as mean ± standard error of the mean, and p values less than 0.05 are considered as statistically significant.

Results

1. SDF-1α Is Actively Secreted by Human Platelets

2. Hoechst-Negative Side Population-Identified Cancer Stem Cell Invasion

3. CD133 Surface Expression-Identified Cancer Stem Cell Invasion

4. MMP-2 and MMP-9 Activity in Sorted A549 Cell Populations

5. MMP-2 mRNA Levels in A549 Cell Populations

1. SDF-1α Is Actively Secreted by Human Platelets

The presence and release of SDF-1α in platelet granules has been previously reported by Massberg et al (Massberg et al., 2006). To confirm that platelets actively secrete SDF-1α, the concentrations of SDF-1α in collagen (10μg/ml)-aggregated platelet releasates were compared to those of resting platelet releasates (10μl/ml saline) using ELISA. Compared to resting platelet releasates, collagen-aggregated platelet releasates had a significantly higher concentration of SDF-1α (6.0 ± 6.0pg/ml SDF-1α in resting platelet releasates vs. 189.15 ± 41.15pg/ml; $n = 3$; $p < 0.05$) (Figure 9). All data are reported as mean ± standard error of the mean.

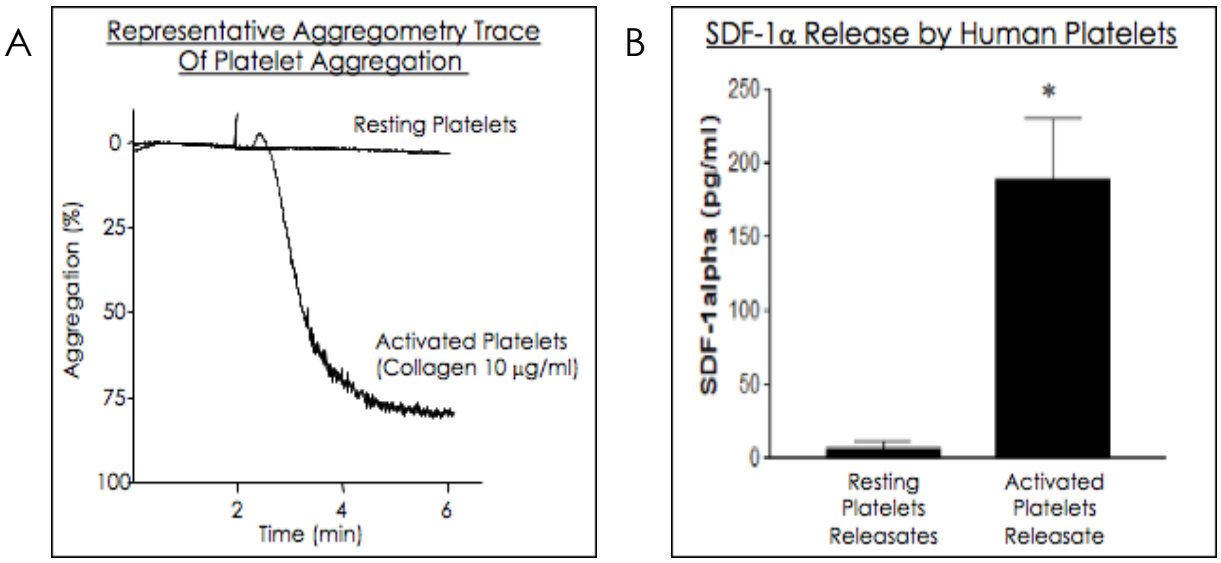


Figure 9: Collagen-aggregated platelet releasates actively release SDF-1α. A. Representative trace of platelet aggregation in response to collagen (10µg/ml) or saline (10µl/ml). B. SDF-1α concentration is analyzed in releasates collected after 6 minutes' aggregation of resting and collagen aggregated platelets. B. SDF-1α concentration in resting platelet releasates vs. collagen-aggregated platelet releasates as measured by ELISA ($n = 3$; $p < 0.05$).

2. Hoechst Negative Side Population-Identified Cancer Stem Cell in A549 Cell Line

To confirm previous reports that platelets stimulate invasion by cancer cells (Alonso-Escolano et al., 2006; Radziwon-Balicka et al., 2014), a single modified Boyden chamber control experiment was carried out to demonstrate a pronounced increase in invasion by A549 cells in response to collagen-aggregated platelet releasates compared to vehicle control (Figure 10). Thus, having established that platelets stimulate lung cancer cell invasion, further experiments were performed to determine whether collagen-aggregated platelet releasates have preferential effects on the invasion by lung cancer CSCs vs. non-CSCs.

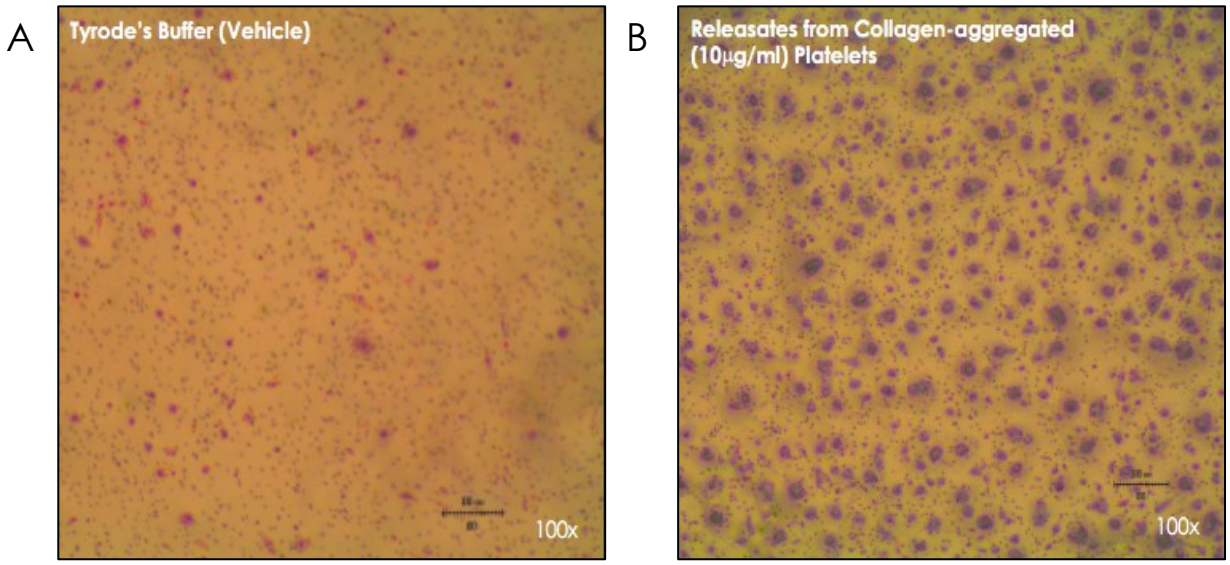


Figure 10: Invasion of A549 cells through a gelatin-coated membrane insert in response to A. Vehicle control (Tyrode's Buffer) vs. B. Collagen (10µg/ml)-aggregated platelet releasate. Invaded cells on the bottom of the inserts were fixed in 4% formaldehyde in PBS and stained with Diff-Quick stained as described previously *(Radziwon-Balicka, Moncada de la Rosa, Zielnik, Doroszko, & Jurasz, 2013).*

2.1 Effect of Resting and Aggregated Platelet Releasate on Invasion by A549 Cancer Stem Cells as Identified by Hoechst Negative Side Population

To determine whether collagen-aggregated platelet releasates have a preferential effect on A549 SP invasion, A549 cells were seeded in a gelatin-coated Boyden chamber and incubated for 24 hours with either collagen-aggregated or resting platelet releasates. The presence of SP cells in the A549 cell line was confirmed using Hoechst 33342 dye (5μg/ml) and Verapamil (50μM) (Figure 11). After 24 hours, a significantly higher proportion of SP cells invaded in response to collagen-aggregated platelet releasates then in response to resting platelet releasates (SP 4.3 ± 0.3% of invaded cells with resting platelet releasates vs. SP 7.6 ± 0.7% of invaded cells with collagen aggregated platelet releasates; $n = 8$; $p < 0.05$) (Figure 12). All data are reported as mean ± standard error of the mean.

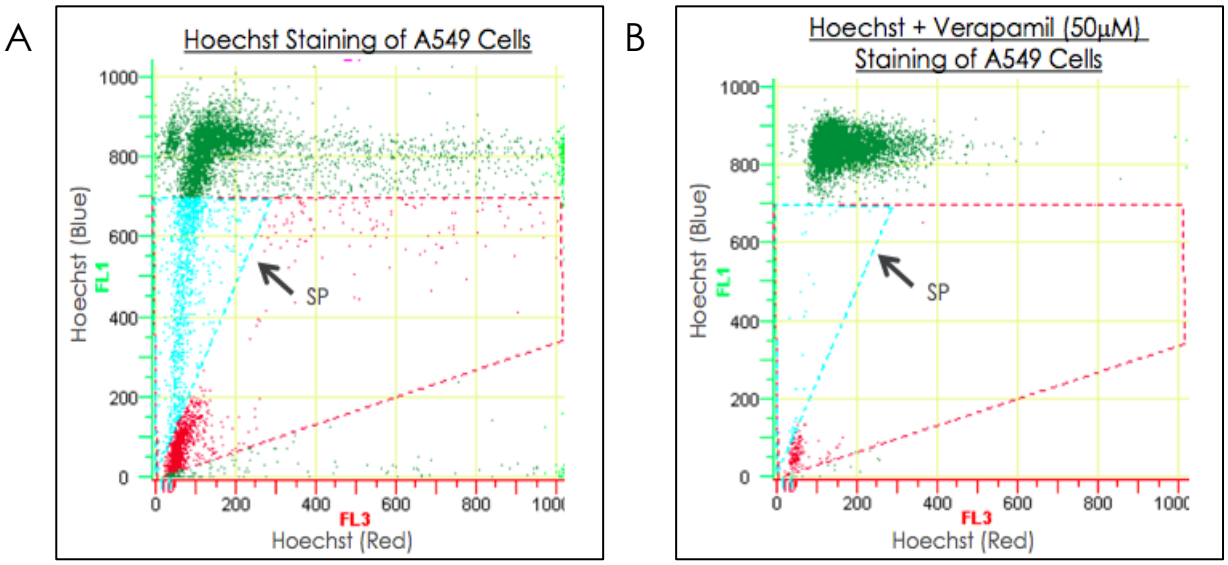


Figure 11: Establishing the Hoechst-negative side population (SP) in the A549 cell line. Representative dot plot from flow cytometry showing: A. The distribution of different populations (blue = Hoechst 33342 SP; green = general A549 population) in A549 cells after staining with Hoechst 33342 (5µg/ml); and B. The addition of 50µM Verapamil, a ABCG2 transporter inhibitor, eliminated the SP.

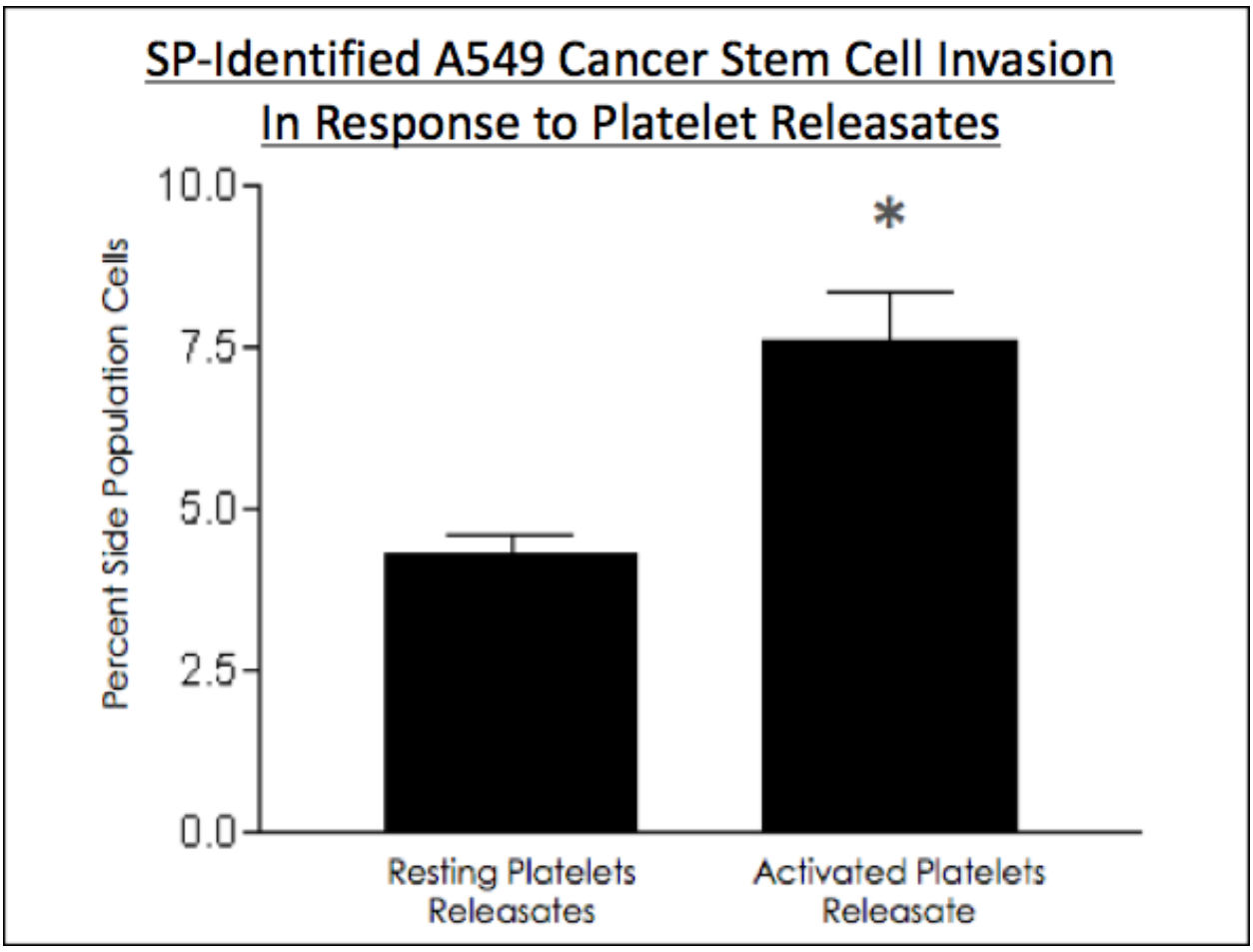


Figure 12: Invasion of A549 cells in response to resting and collagen-aggregated platelets after 24 hours. Summary bar graph of Hoechst 33342 negative SP identified CSC invasion in response to either resting or activated platelets ($n = 8$; $p < 0.05$).

2.2 Effect of CXCR-4 & MMP Inhibitors on Invasion by A549 Side Population in Response to Aggregated Platelet Releasates

Before the invasion assays were performed, Annexin V experiments were first performed to determine whether the inhibitors affect A549 cell viability at their effective concentrations. The MMP inhibitor phenanthroline caused significant apoptosis in the A549 cells at the working concentration, as compared to control (16.95 ± 6.18% apoptosis in response to DMEM (control), 16.78 ± 6.16% apoptosis in response to AMD3100 (10μM), 87.65 ± 4.03% apoptosis in response to Phenanthroline (1mM) and 32.31 ± 8.94% apoptosis in response to GM6001 (10μM), after 24-hours; $n = 4$; $p < 0.05$, compared to control) (Figure 13). Therefore, only AMD3100 (10μM) and GM6001 (10μM) was used in future experiments.

To identify whether the preferential increase in Hoechst-negative SP-identified CSC invasion stimulated by collagen-aggregated platelet releasates was due to the SDF-1α: CXCR-4: MMP signalling, the Boyden chamber invasion assay was performed in the presence of the CXCR-4 antagonist AMD3100 and the MMP inhibitor GM6001.

The invasion of A549 SP cells through gelatin-coated membrane in response to collagen-aggregated platelet releasates was not affected by AMD3100 or GM6001 (25.15 ± 8.04% SP invasion in the presence of AMD3100 and 20.56 ± 8.10% SP invasion with GM6001 compared to 21.38 ± 7.86% SP invasion in groups with no treatment (control); $n = 12$, $p = 0.19$) (Figure 14. A). However, AMD3100 was able to decrease the number of total A549 cell invasion (44.23 ± 9.88 x 10^3 cells invaded in AMD3100 treated samples vs. 60.85 ± 13.37 x 10^3 cells invaded in

control samples; $n = 13$, $p < 0.05$) (Figure 14. B). All data are reported as mean ± standard error of the mean.

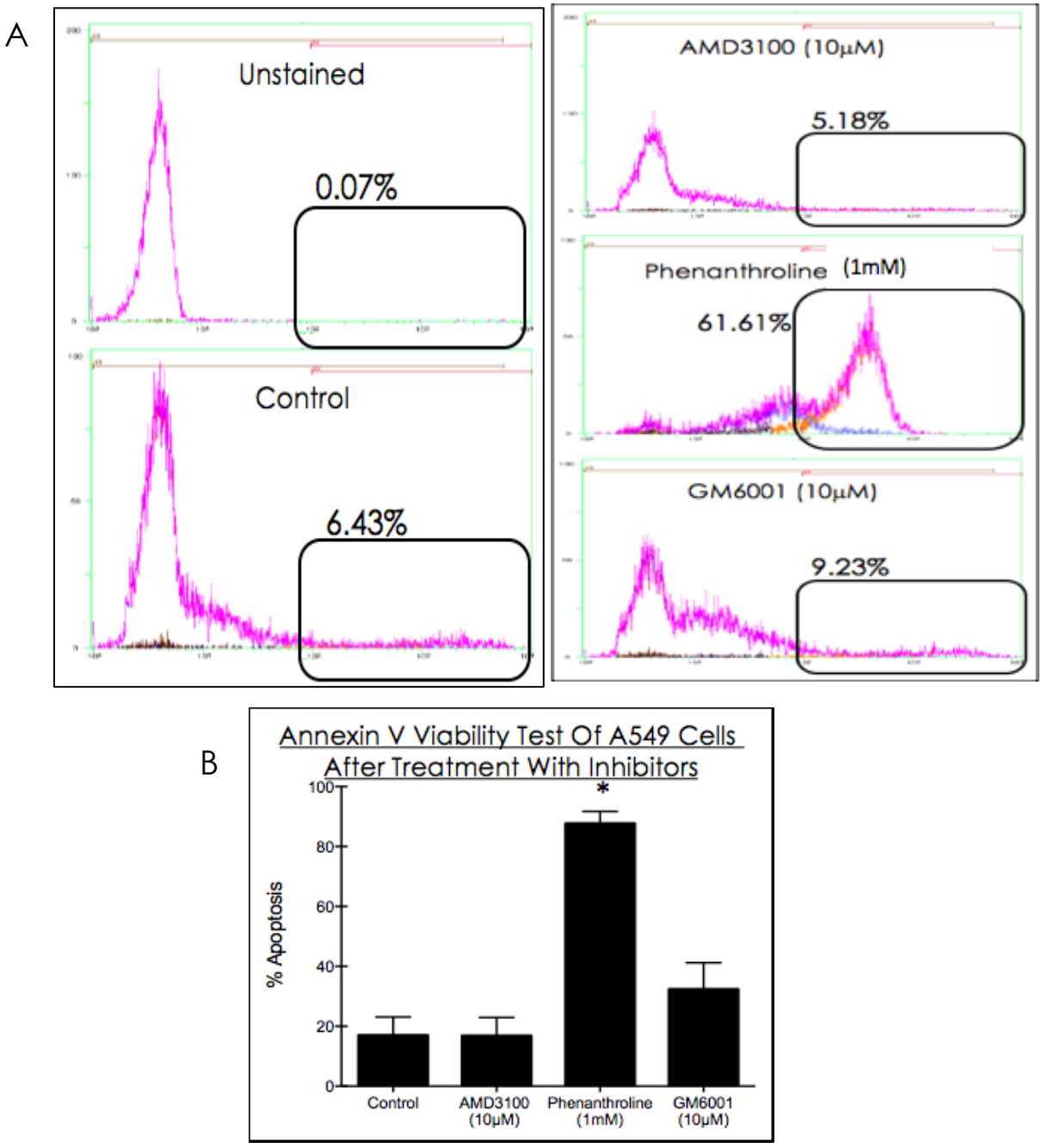


Figure 13: Annexin V apoptosis test for SDF-1alpha: CXCR-4: MMP signalling pathway inhibitors after 24hr incubation. A. Representative histogram from flow cytometry and B. Summary bar graph of A549 cell viability after 24-hour treatment with AMD3100 (10µM), Phenanthroline (1mM) and GM6001 (10µM) compared to control (DMEM) ($n = 4$, $p < 0.05$).

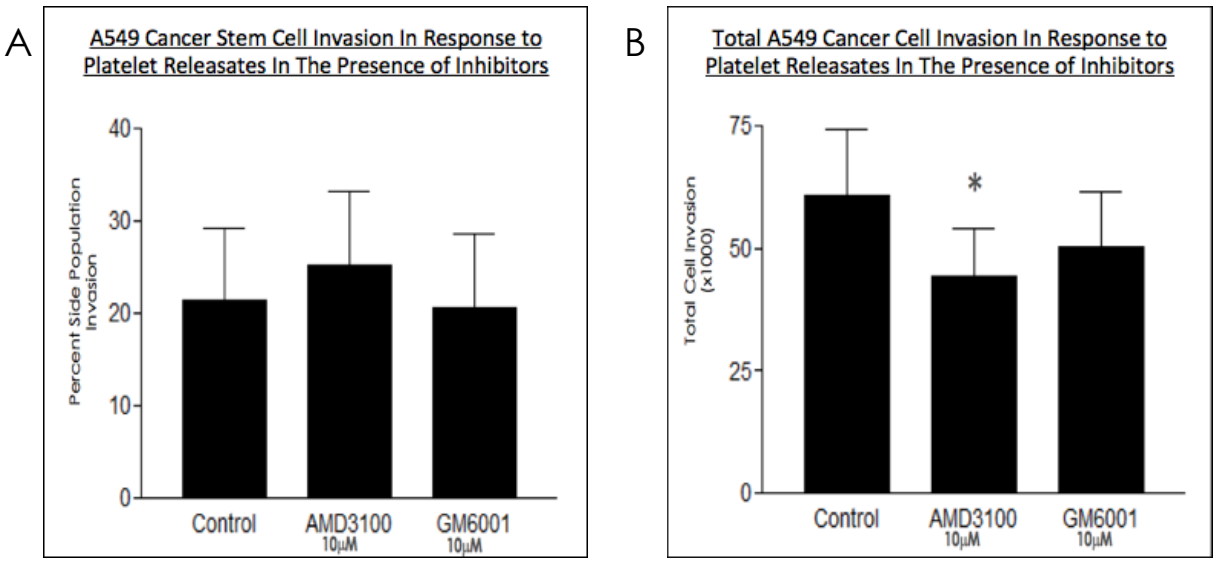


Figure 14: 24hr invasion of SP identified A549 CSC invasion through gelatin coated PVDF membrane. Summary bar graph of A. Hoechst 33342 negative SP identified CSC (n = 12, p =0.19, compared to control), and B. Total A549 cell invasion in response to collagen-aggregated platelet releasates in the presence of either AMD3100 (10μM) or GM6001 (10μM) (n = 13, $p < 0.05$, compared to control).

3. Comparison of the Effects of Platelets on CSC vs. Non-CSC Invasion as Identified by CD133 Staining

Hoechst 33342 is useful in the identification of stem and cancer stem cells through the excretion of the dye by ABCG2 from these cells. However, many tumor cells also express ABCG2 transporters, which contributes to their drug resistance ability (Chen et al., 2011). Since tumors are known for their heterogeneity, I hypothesized that non-CSC cells within the A549 cell line may also express higher levels of these transporters and as a result, using Hoechst 33342 as an identification of CSCs may not be sufficiently accurate. CD133 is a transmembrane glycoprotein has been extensively utilized as a means of identifying stem and progenitor cells as well as CSCs among many different cell lines (Sahlberg, Spiegelberg, Glimelius, Stenerlow, & Nestor, 2014). More importantly, there are multiple reports confirming the expression of CD133 in the A549 cell line (Roudi, Madjd, Ebrahimi, Samani, & Samadikuchaksaraei, 2014; H. Zhang et al., 2014; H. Z. Zhang et al., 2010).

3.1 Detection of CD133 Expressing Subpopulation in A549 Human Lung Carcinoma Cell Line

To confirm that the A549 cell line contains a CD133 expressing subpopulation, as well as to determine what proportion of Hoechst 33342-negative SP express the CD133 marker, both total A549 cells and the Hoechst 33342-negative SP were stained for CD133 surface expression. The flow cytometry results demonstrated that a small percentage of A549 cells are indeed positive for CD133 (1.45 ± 0.59% CD133-positive cells in total A549 population). Moreover, the Hoechst-negative SP is enriched with CD133-positive cells compared to the general population, although the percentage of SP cells that are positive for CD133 are still relatively small (3.84 ± 1.09% in Hoechst-negative SP; $n = 4$, $p < 0.05$) (Figure 15). All data are reported as mean ± standard error of the mean.

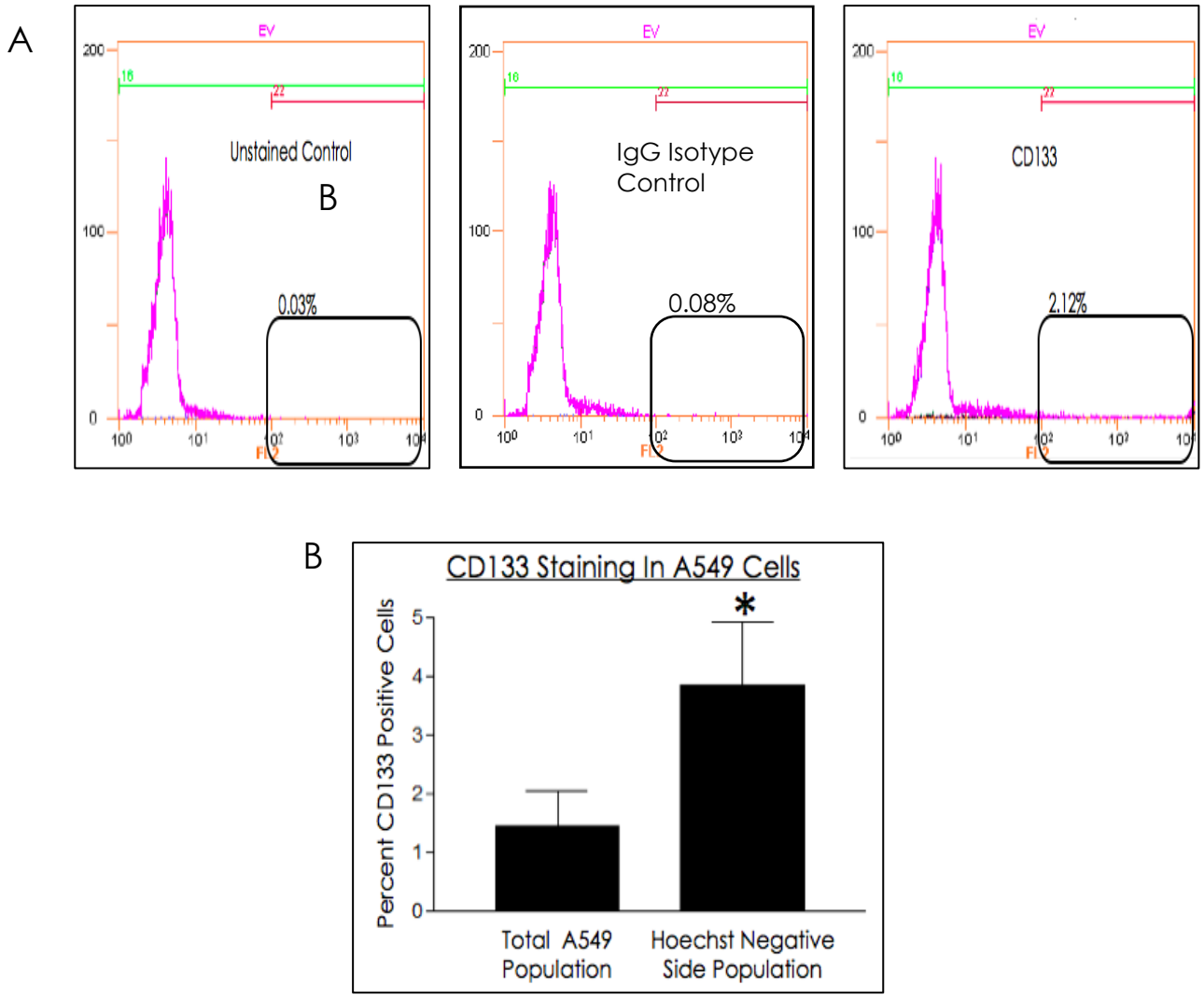


Figure 15: CD133 surface expression by A549 cells. A. Representative histogram of CD133 expression from flow cytometry and B. Summary bar graph of CD133 expression in total and Hoechst-negative SP of the A549 cell line ($n = 4$, $p < 0.05$).

3.2 CD133 Staining Identified Cancer Cell Invasion in Response to Resting and Activated Platelet Releasates

To determine whether collagen-aggregated platelet releasates have a preferential effect on the invasion of CD133-identified CSCs in the A549 cell population, the invasion assays in gelatin-coated Boyden chambers were repeated as previously described. However, due the extreme low number of CD133 expressing A549 cells, it was difficult to identify CD133-positive cells via antibody staining of the invaded cells. Hence, an alternate approach was pursued. A549 cells were first sorted based on the expression of the CD133 marker using FACS, and the enrichment and purity of the CD133-positive subpopulation was assessed (3.56 ± 0.76% CD133-positive cells in the un-sorted samples vs. 55.99 ± 6.50% of CD133-positive cells in the CD133-positive subpopulation after sorting; $n = 9$, $p < 0.05$) (Figure 16).

Having established that the FACS was able to isolate and enrich the CD133-positive A549 subpopulation, A549 cells were sorted into CD133-positive and CD133-negative fractions for use in invasion assays. Following the sort, an equal number of cells from either fraction was stained with cell tracker dyes. As control experiments, the effect of the cell tracker stains on A549 cell survival at their working concentration was assessed by Trypan Blue staining. Neither cell tracker dye induced significant cell death when compared to no treatment (88.33 ± 2.73% cells viable after 24-hour incubation with DMEM vs. 89.33 ± 3.48% viable after 24-hour incubation with CMFDA (5μM) vs. 89.33 ± 2.33% cell viable after 24-hour incubation with CMTPX (5μM); $n = 3$, $p = 0.81$) (Figure 17). All data are reported as mean ± standard error of the mean.

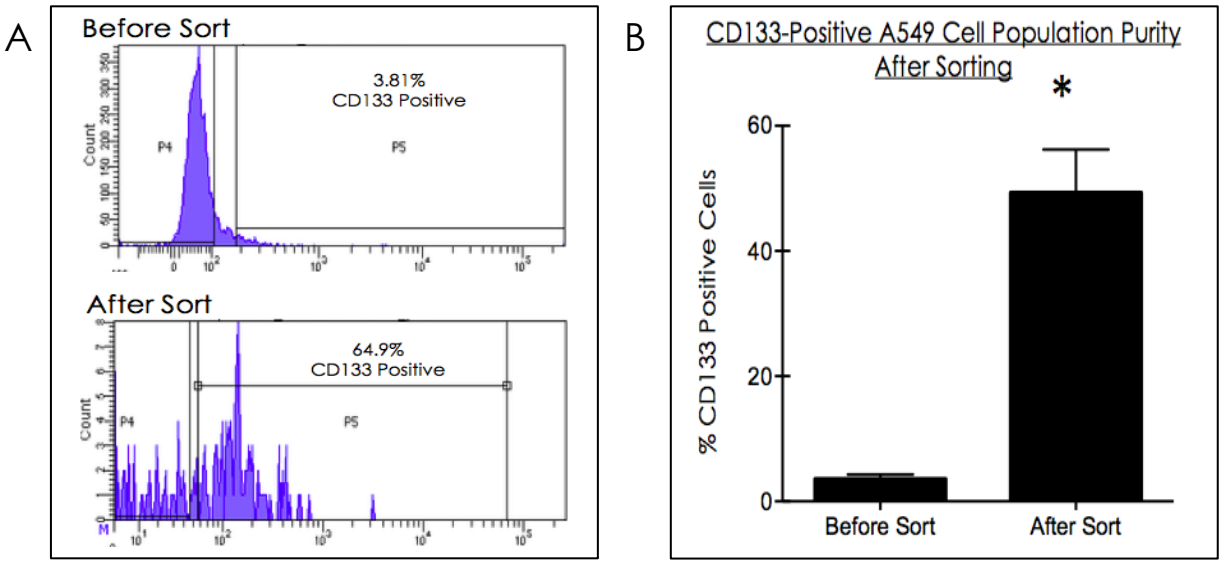


Figure 16: CD133- based FACS in A549 cells. A. Representative histogram from flow cytometry and B. Summary bar graph of CD133-positive population purity before and after cell sort based on CD133 positivity.

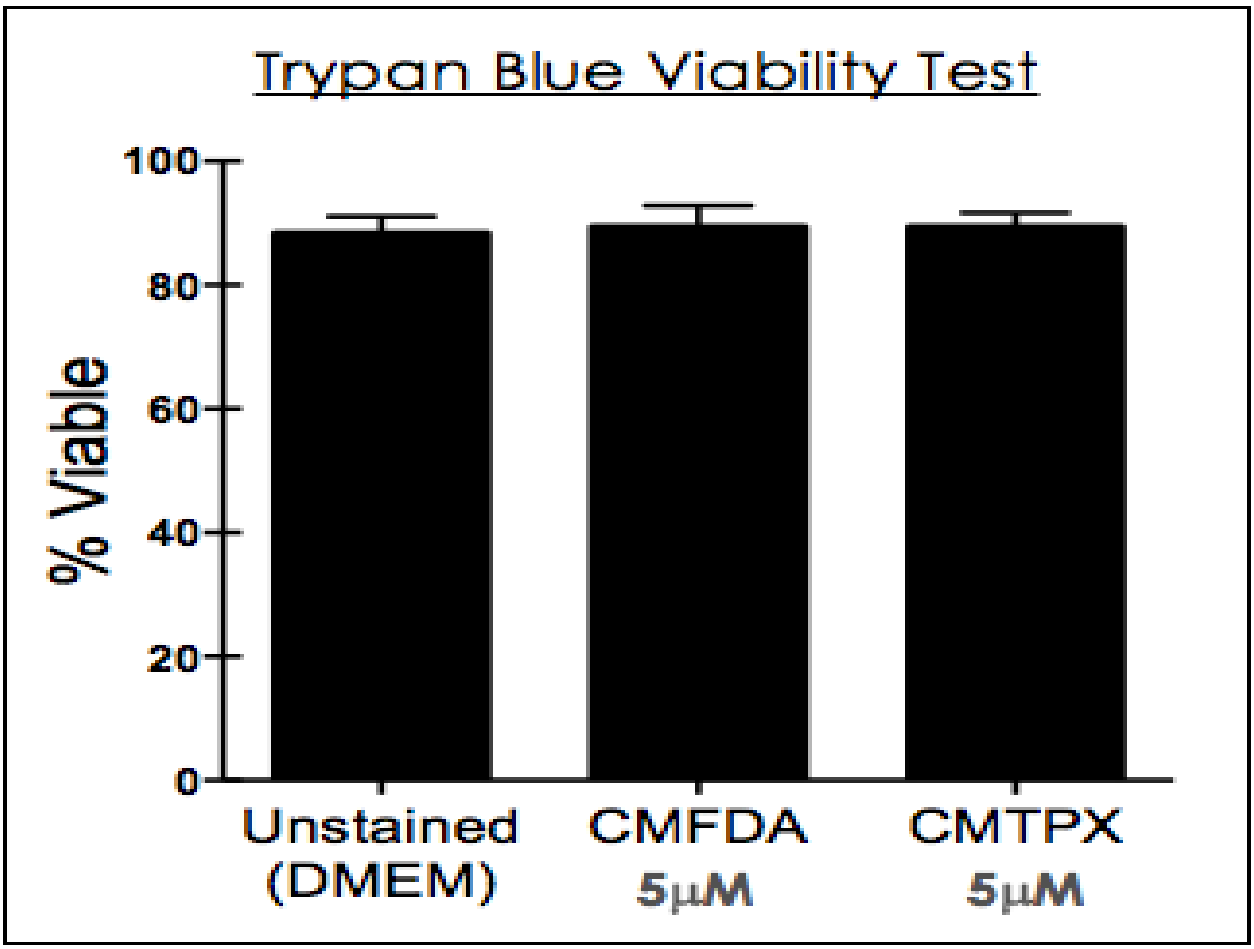


Figure 17: Summary bar graph of Trypan Blue viability experiment of A549 cells after cell tracker dye staining (n = 3, p = 81).

For initial invasion assays, the CD133-positive fraction was stained with the green CMFDA cell tracker dye (5μM) and the CD133-negative fraction was stained with the red CMTPX dye (5μM). The two populations were combined after staining to perform the invasion assay in the gelatin-coated Boyden chambers. After 24 hours, the membrane in the chamber was removed and imaged under a fluorescence confocal microscope (Figure 18).

A significantly higher number of CD133-positive cells invaded in response to both resting and collagen-aggregated platelet releasates (Resting releasate: 39.29 ± 2.30% CD133-negative cells vs. 60.71 ± 2.30% CD133-positive cells; $n = 7$; $p < 0.05$) (Aggregated releasates: 32.86 ± 4.20% CD133-negative cells vs. 67.14 ± 4.20% CD133-positive cells; $n = 7$, $p < 0.05$) (Figure 19. A). This indicate CD133-positive A549 cells are more invasive then CD133-negative A549 cells. Further analysis showed that the percent invasion by CMTPX-stained CD133-negative cells decreased when comparing invasion in response to resting vs. collagen-aggregated releasates, while CMFDA-stained CD133-positive A549 cell invasion increased from resting to collagen-aggregated platelet releasates (CD133-negative cell: 39.29 ± 2.30% change with resting vs. 32.86 ± 4.18% change with aggregated platelet releasate; $n= 7$, $p = 0.09$) (CD133-positive cell: 60.71 ± 2.30% change with resting vs. 67.14 ± 4.18% change with aggregated platelet releasates; $n = 7$, $p = 0.09$) (Figure 19. B). This suggests that activated platelet releasates preferentially stimulate the invasion of CD133-identified CSCs, which is consistent with data from the Hoechst-negative SP-identified CSCs invasion assays.

To control for any potential effect of the cell tracker dyes on A549 cell invasion, the same experiments were repeated with CMTPX-stained CD133-positive and CMFDA-stained

CD133-negative A549 subpopulation. Upon performing the reverse experiments, similar results were obtained, suggesting the cell tracker dyes did not have an effect on A549 cell invasion (Resting releasates: 37.00 ± 3.45% CD133-negative cells vs. 63.00 ± 3.45% CD133-positive cells; $n = 5$; $p < 0.05$) (Activated releasates: 32.60 ± 4.46% CD133-negative cells vs. 67.60 ± 4.32% CD133-positive cell; $n = 5$; $p < 0.05$) (CD133-negative cell: 37.00 ± 3.45% change with resting vs. 32.60 ± 4.46% change with aggregated platelet releasate; $n= 7$, $p = 0.28$) (CD133-positive cell: 63.00 ± 3.45% change with resting vs. 67.60 ± 4.32% change with aggregated platelet releasates; $n = 7$, $p = 0.25$) (Figure 20). All data are reported as mean ± standard error of the mean.

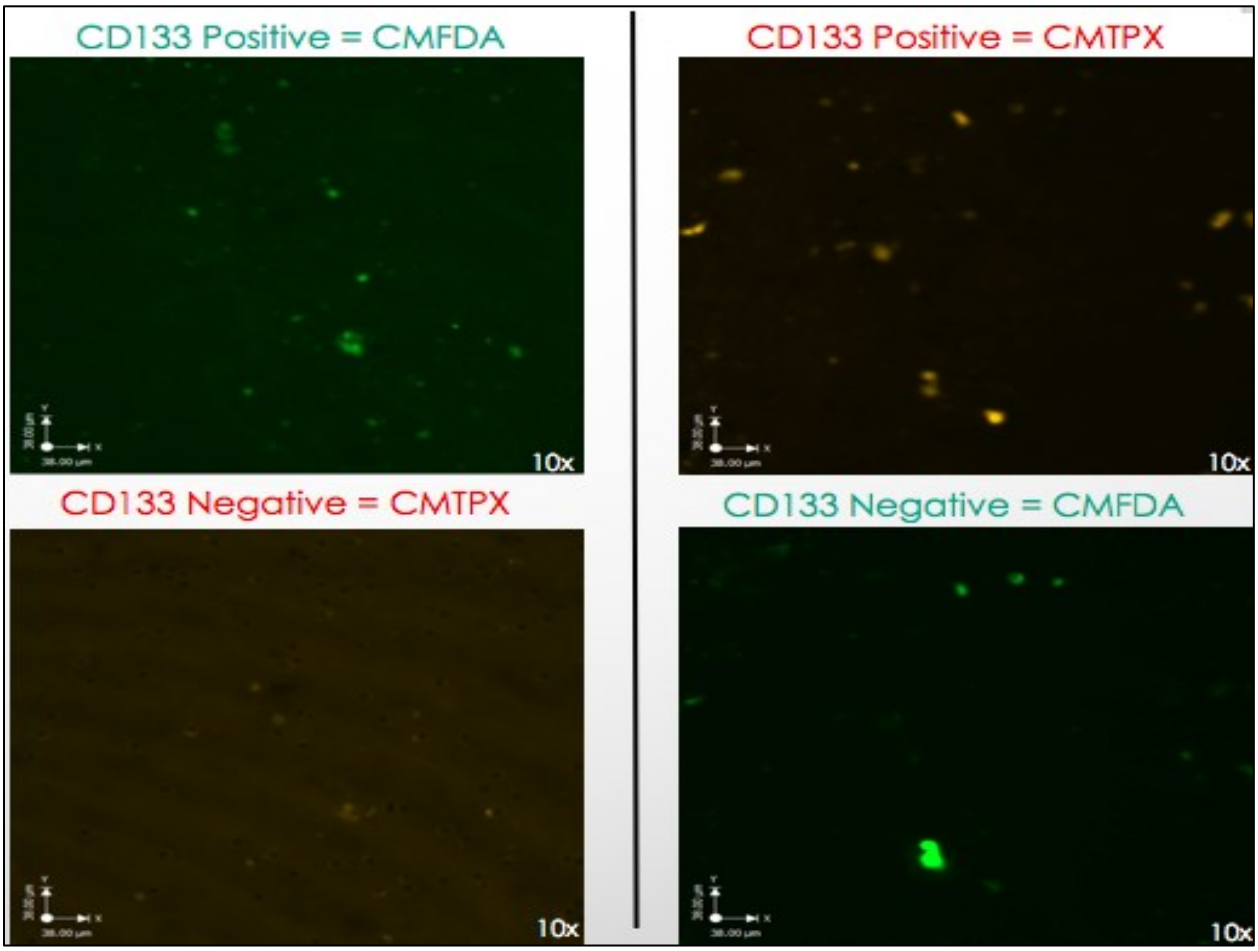
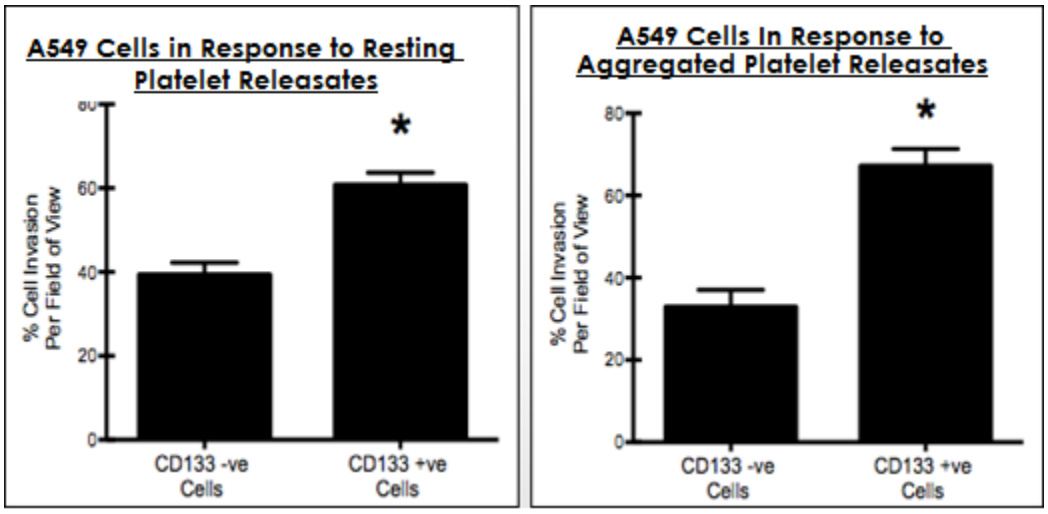


Figure 18: Representative confocal microscopy image of cell tracker stained A549 CD133-positive and CD133-negative populations after the 24-hour invasion assay.

A



B

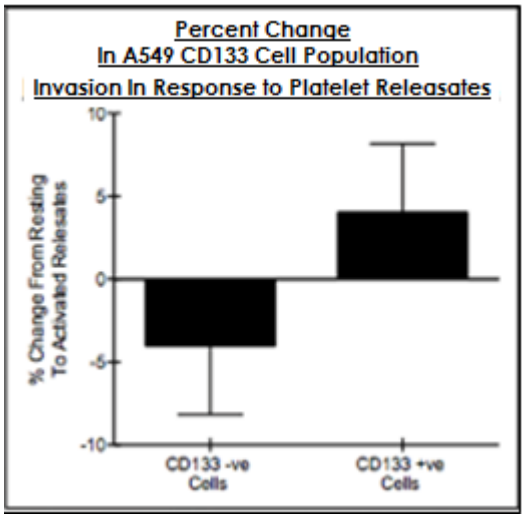
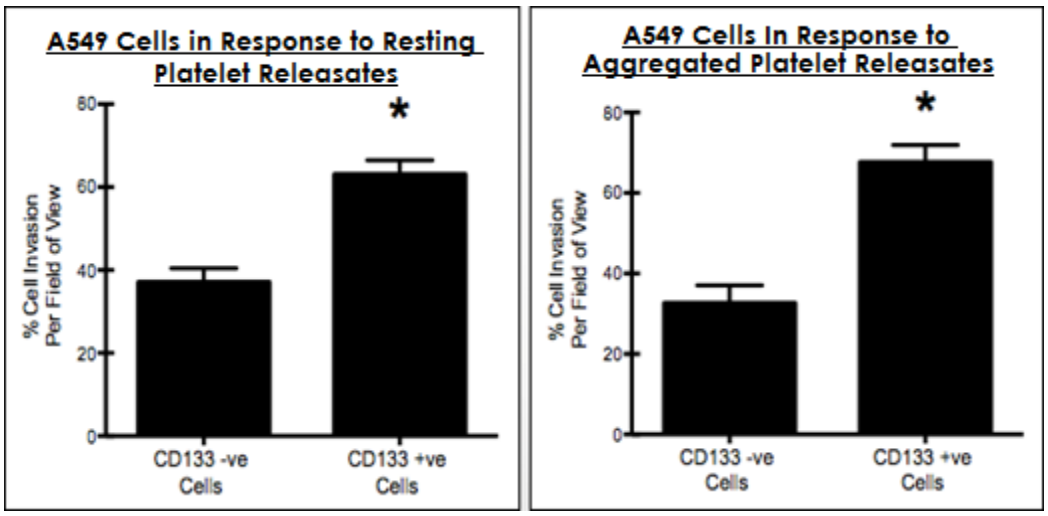


Figure 19: CD133-identified CSC invasion. A. Summary bar graph comparing the invasion (24hr) of CMFDA stained-CD133-positive and CMTPX stained-CD133-negative A549 cells in response to resting or collagen-aggregated platelet releasate. Changes in each population between resting and collagen-aggregated platelet releasates are shown in B (A. $n = 7$, $p < 0.05$; B. $n = 7$, $p = 0.19$).

A



B

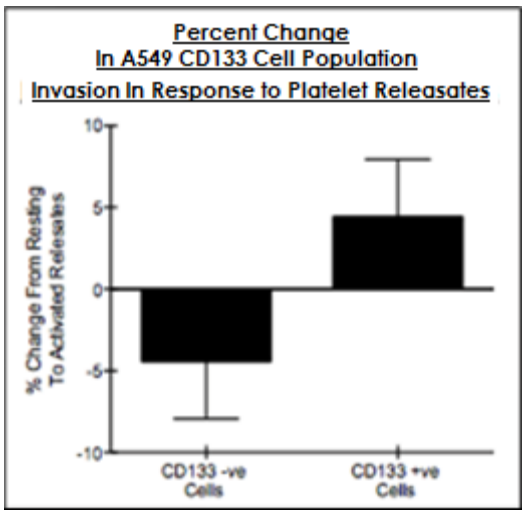


Figure 20: CD133-identified CSC invasion (reverse experiment). A. Summary bar graph comparing the invasion of CMTPX stained-CD133-positive and CMFDA stained-CD133-negative A549 cells in response to resting or collagen-aggregated platelet releasate. Changes in each population between resting and collagen-aggregated platelet releasates are shown in B (A. $n = 5$, $p < 0.05$; B. $n = 5$, $p = 0.14$).

3.3 Effects of Washed Platelet and Platelet Releasates on CD133 Expression by A549 Cells

It has been reported that platelets can stimulate cancer cells to undergo EMT to gain a stem cell-like phenotype and motility, thus allowing them to become more invasive (Son & Moon, 2010). To investigate whether the preferential increase in CD133-positive A549 cell invasion in response to aggregated platelet releasates was due to rue invasion by CD133-positive A549 cells and not simply due to a platelet-induced EMT program that converted CD133-negative A549 into CD133-positive A549, the surface expression of CD133 by A549 cells before and after treatment with both platelet releasates and isolated platelets was compared. Incubation with either platelet releasates or isolated platelets caused no significant change in A549 cell CD133 surface expression (0.99 ± 0.31% CD133-positive cells in untreated A549 population vs. 0.75 ± 0.17% CD133-positive cells in A549 population treated with resting platelet releasate vs. 0.78 ± 0.20% CD133-positive cells with activated platelet releasate vs. 0.86 ± 0.28% CD133-positive with washed platelets; $n = 3$, $p = 0.10$, compared to untreated cells). This supports the hypothesis in that the preferential stimulation of CD133-positive cell population invasion was due to an effect on the cells already expressing CD133 and not due to platelets causing an increase in CD133 expression by A549 cells (Figure 21). All data are reported as mean ± standard error of the mean.

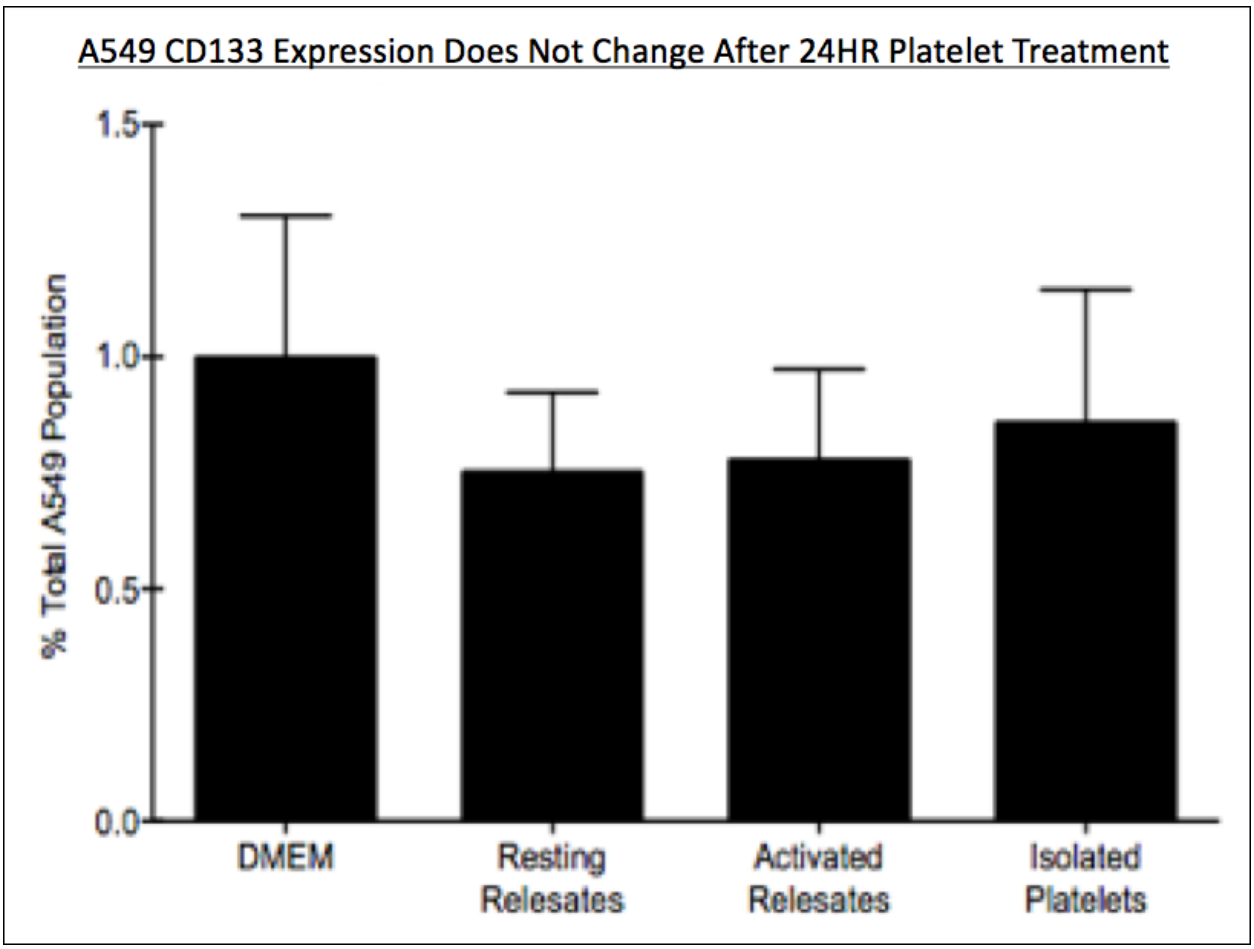


Figure 21: A549 cell CD133 surface expression in response to human platelets. Summary bar graph of A549 cell CD133 expression in response to 24-hour treatment with DMEM (control), resting platelet releasates, collagen-aggregated platelet releasates and washed platelets (n = 3, p = 0.10, compared to untreated cells (DMEM)).

3.4 CD133 Surface Expression Identified Cancer Cell Invasion in Response to CXCR-4 & MMP Inhibitors

Inhibitors of SDF-1α signalling and MMP activity were added to identify whether the preferential effect of collagen-aggregated platelet releasates on CD133-positive A549 cell population invasion was dependent on SDF-1α: CXCR-4: MMP signalling via pharmacological means. AMD3100 is a small molecule inhibitor of the CXCR-4 receptor, and works by blocking the binding site of CXCR-4's endogenous ligand – SDF-1α (Rosenkilde et al., 2004). GM6001 is a reversible broad-spectrum inhibitor of MMPs and its mechanism of action depends on the chelation of the central zinc in the active site of MMPs (Ikejiri et al., 2005).

Contrary to the invasion assays studying Hoechst-negative SPs in the A549 cell line, the addition of neither AMD3100 (10μM) nor GM6001 (10μM) to the invasion assay significantly decreased the total number of cells that invaded in response to collagen-aggregated platelet releasates when compared to control (58.90 ± 5.29% of control invaded with AMD3100 vs. 83.07 ± 17.60% of control invaded with GM6001; $n = 3$, $p = 0.086$) (Figure 22). All data are reported as mean ± standard error of the mean.

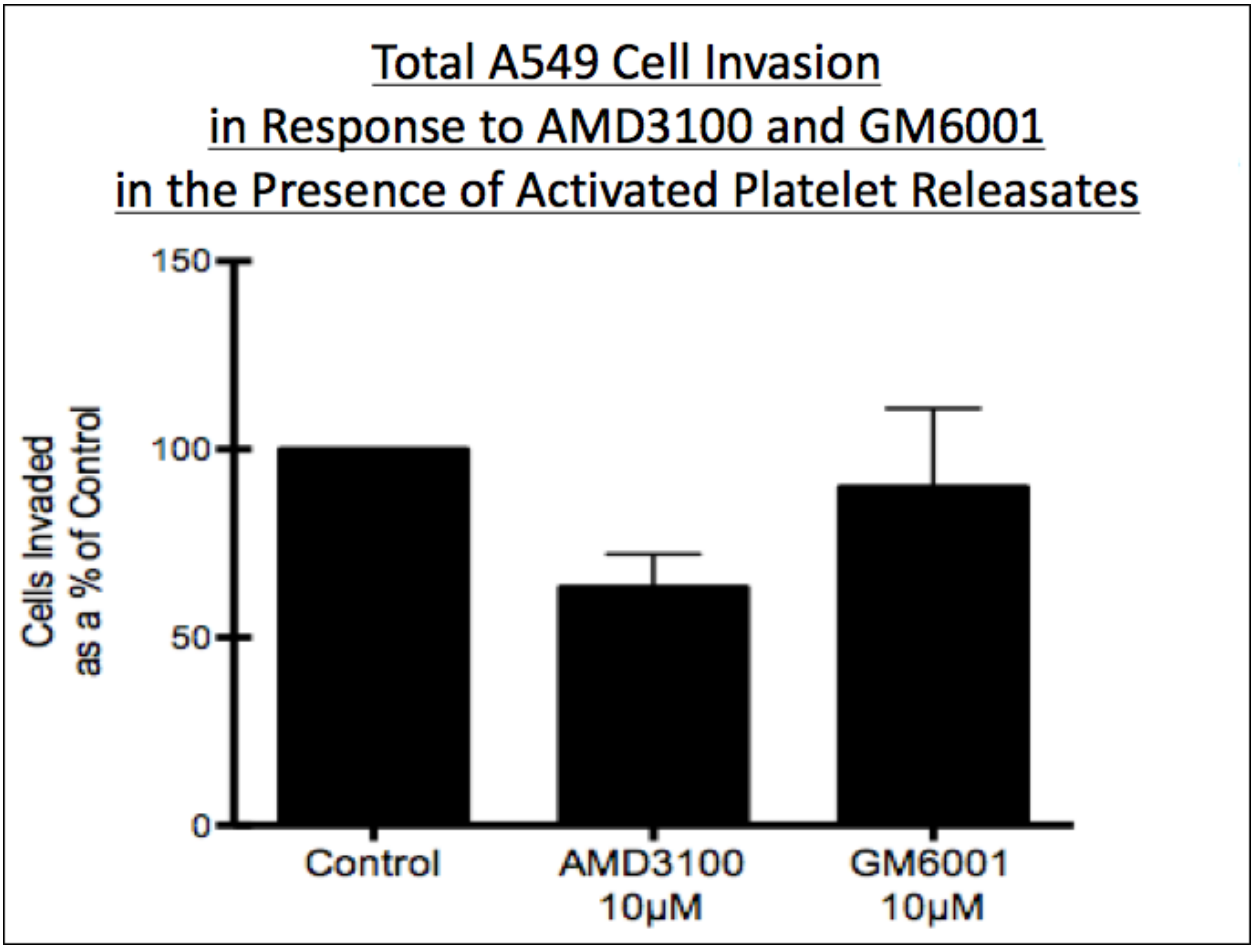


Figure 22: Total A549 cell invasion (24hr) in response to AMD3100 and GM6001. Summary bar graph of total A549 cell invasion in response to 24-hour treatment with collagen-aggregated human platelet releasate in the presence of either AMD3100 (10μM) or GM6001 (10μM) (n = 3, p =0.086).

Next, the effect of AMD3100 and GM6001 on the invasion of CD133-positive and CD133-negative subpopulations in the A549 cell line in response to collagen-aggregated platelet releasates were investigated. Compared to control, the addition of AMD3100 and GM6001 did not induce a statically significant decrease in CD133-negative cells that have invaded (102.3 ± 39.45% invasion of control in the presence of AMD3100 (10μM) and 158.3 ± 56.95% of control in the presence of GM6001 (10μM); $n = 3$, $p = 0.54$, compared control (no treatment)) (Figure 23. A). However, both AMD3100 and GM6001 was able to decrease the number of invaded CD133-positive cells in response to collagen-aggregated platelet releasates (50.67 ± 3.48% invasion of control in the presence of AMD3100 (10μM) and 66.67 ± 9.56% invasion of control in the presence of GM6001 (10μM); $n = 3$, $p < 0.05$, compared control (no treatment)) (Figure 23. B). All data are reported as mean ± standard error of the mean.

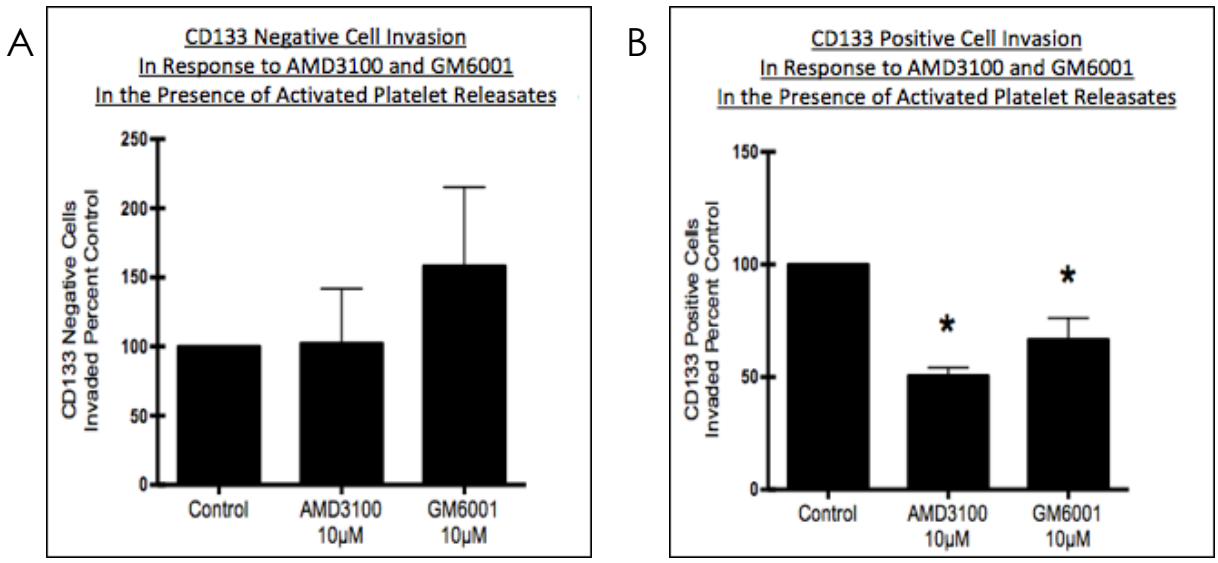


Figure 23: CD133-negative vs. CD133-positive cell invasion (24hr) in response to AMD3100 and GM6001. Summary bar graph of CD133-negative vs CD133-positive cell invasion in response to collagen-aggregated human platelet releasate in the presence of either AMD3100 (10μM) or GM6001 (10μM) (A. $n = 3$, $p = 0.54$; B. $n = 3$, $p < 0.05$, compared to control).

4. Analysis of MMP-2 & MMP-9 Levels in A549 Cells Sorted Based on CD133 Surface Expression

4.1 Gelatin Zymography of MMP-2 and MMP-9 Activity in CD133-negative and CD133-positive A549 cells

To study the effect of direct platelets on MMP-2 and MMP-9 activity in A549 CD133-positive and CD133-negative subpopulations, the A549 cells were first sorted based on CD133 surface expression and seeded. Washed human platelets were then added to the sorted A549 cells 24 hours before zymography was performed. Gelatin zymography showed that when compared to CD133-negative cells, CD133-positive cells had higher basal levels of MMP-2 activity (37.87 ± 15.05 arbitrary units of gelatinolytic activity representing MMP-2 activity in CD133-negative cell vs. 53.77 ± 17.02 arbitrary units in CD133-positive cells, corrected to β-actin; $n = 16$, $p < 0.05$) (Figure 24. A & B). After 24-hour treatment with washed human platelets, there was an increase in MMP-2 proteins in the CD133-negative populations, but not in CD133-positive populations ((1.71 ± 0.78 arbitrary units of MMP-2 gelatinolytic activity in CD133-negative cell vs. 1.60 ± 0.75 arbitrary units in CD133-positive cells, corrected to β-actin; $n = 6$, $p = 0.70$) (Figure 24. C & D).

MMP-9 levels were minimal in both populations and were not affected significantly by platelet co-incubation (Before treatment: 1.65 ± 1.71 arbitrary units representing MMP-9 gelatinolytic activity in CD133-negative cell vs. 2.25 ± 1.56 arbitrary units in CD133-positive cells, corrected to β-actin; $n = 6$, $p = 0.40$. After treatment: 1.30 ± 0.77 arbitrary units

representing MMP-9 gelatinolytic activity in CD133-negative cell vs. 1.14 ± 0.70 arbitrary units in CD133-positive cells, corrected to β-actin; n = 6, p = 0.40) (Figure 25). All data are reported as mean ± standard error of the mean.

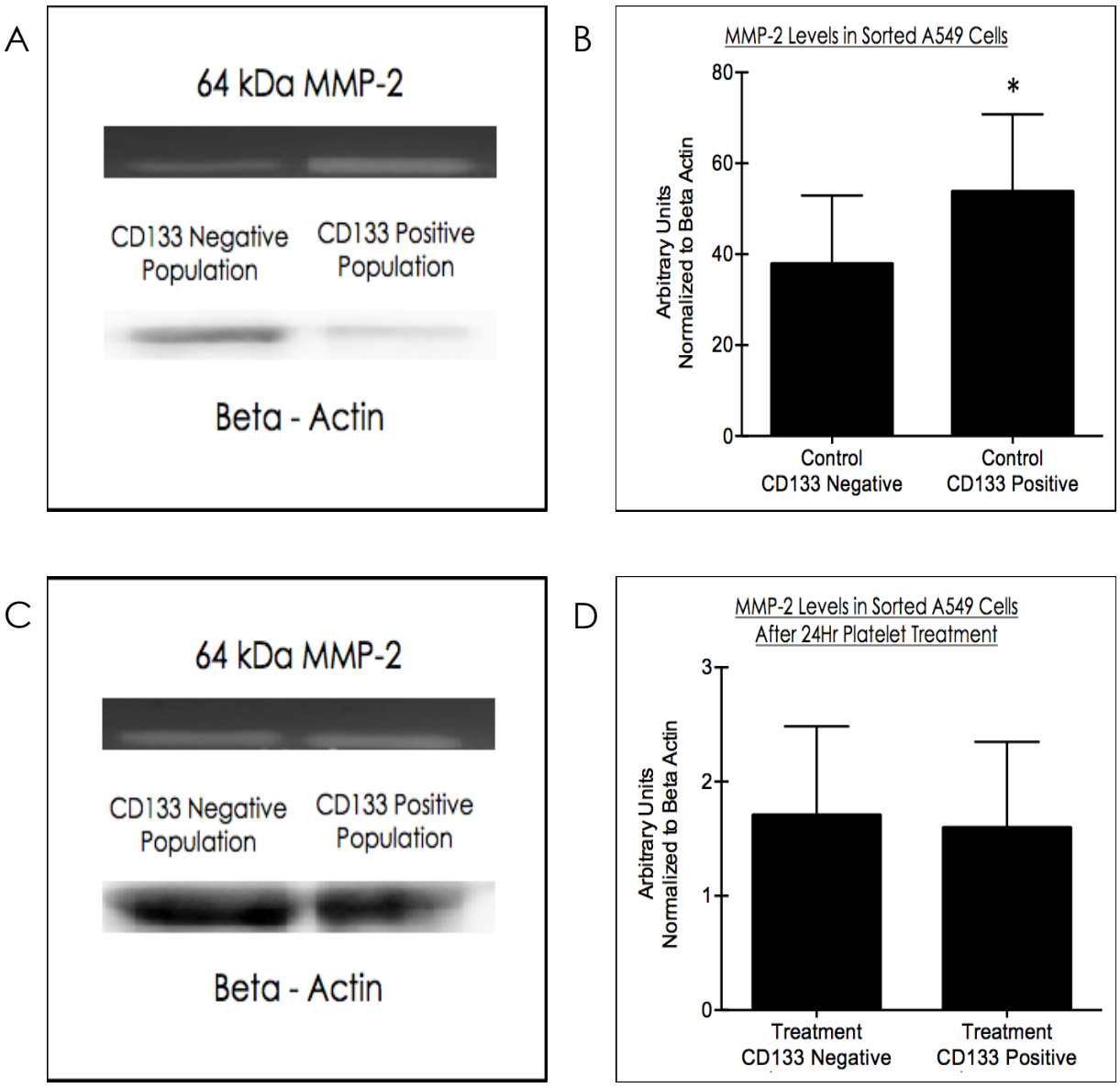


Figure 24: MMP-2 levels in A549 cells. A. Representative zymography and B. Summary bar graph of basal MMP-2 levels in FACS-sorted A549 cells ($n = 16$, $p < 0.05$). C & D are representative zymography and summary bar graph of A549 cell MMP-2 expression after 24-hour treatment with washed human platelets in CD133-positive and CD133-negative A549 cell populations ($n = 6$, $p = 0.70$).

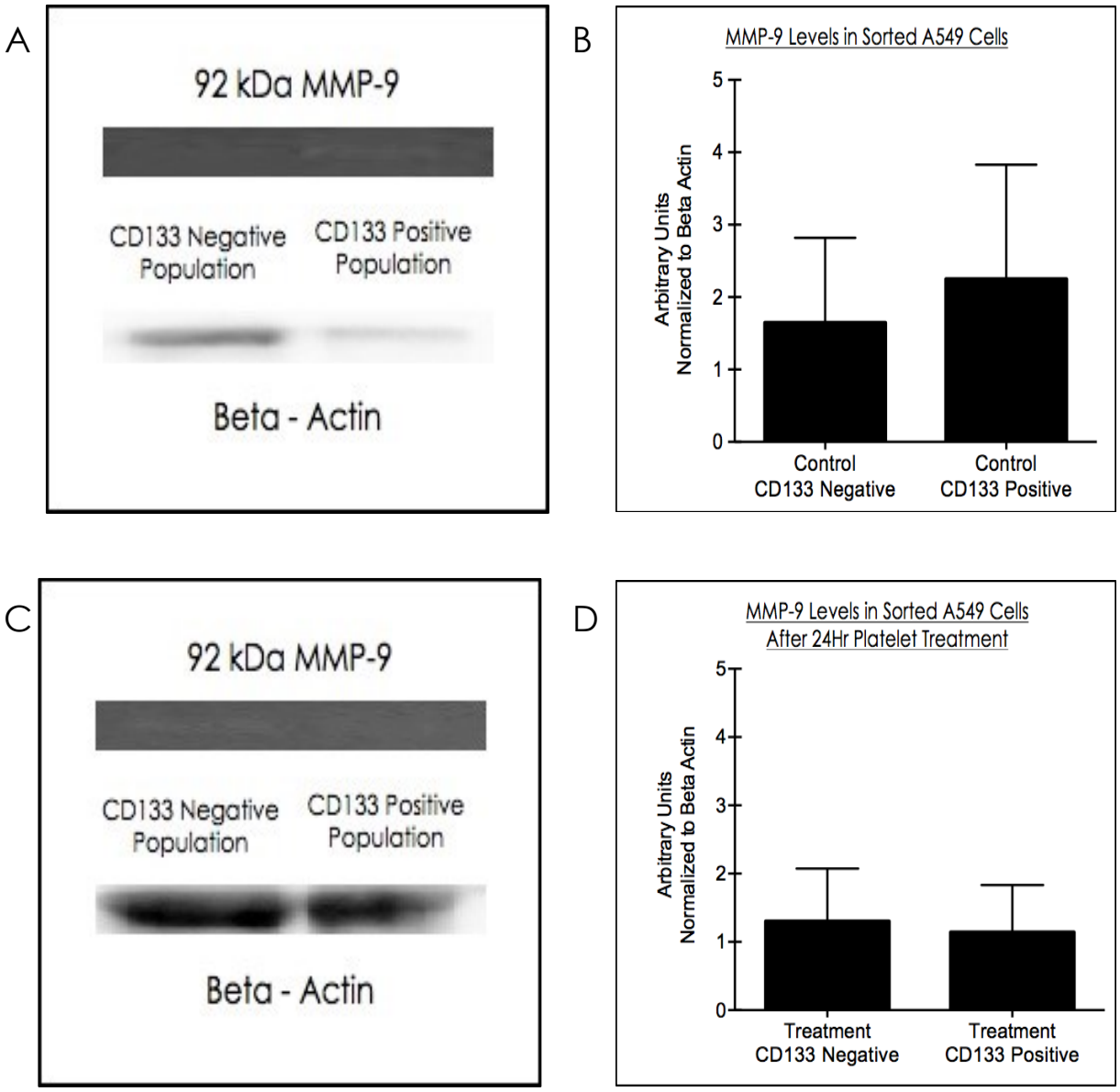


Figure 25: MMP-9 levels in A549 cells. A. Representative zymography and B. Summary bar graph of basal MMP-9 levels in FACS-sorted A549 cells (n = 6, p = 0.40). C & D are representative zymography and Summary bar graph of A549 cell MMP-9 expression after 24-hour treatment with washed human platelets in CD133-positive and CD133-negative A549 cell populations (n = 6, p = 0.40).

4.2 Real Time PCR

Since MMP-2 is found in platelet cytosol, it was difficult to distinguish whether the increase in MMP-2 protein in the previous experiments was due to the addition of platelets or was the result of a transcriptional up-regulation in MMP-2 expression by the A549 cells. Thus, qPCR was conducted on A549 cells that were sorted based on CD133 surface expression and treated with washed human platelets for 24 hours. Platelets do not contain a nucleus and contain minimal mRNA (Rowley, Schwertz, & Weyrich, 2012). Thus, changes in MMP-2 expression levels would be within A549 cells and not from contributing platelets mRNA. Platelet co-incubation significantly increased MMP-2 mRNA levels in the CD133-negative, but not the CD133-positive A549 subpopulations (CD133-negative cells: 9.64 ± 3.9-fold change in CD133-negative cell MMP-2 mRNA levels in response to platelet treatment; $n = 5$, $p < 0.05$) (Figure 26). All data are reported as mean ± standard error of the mean.

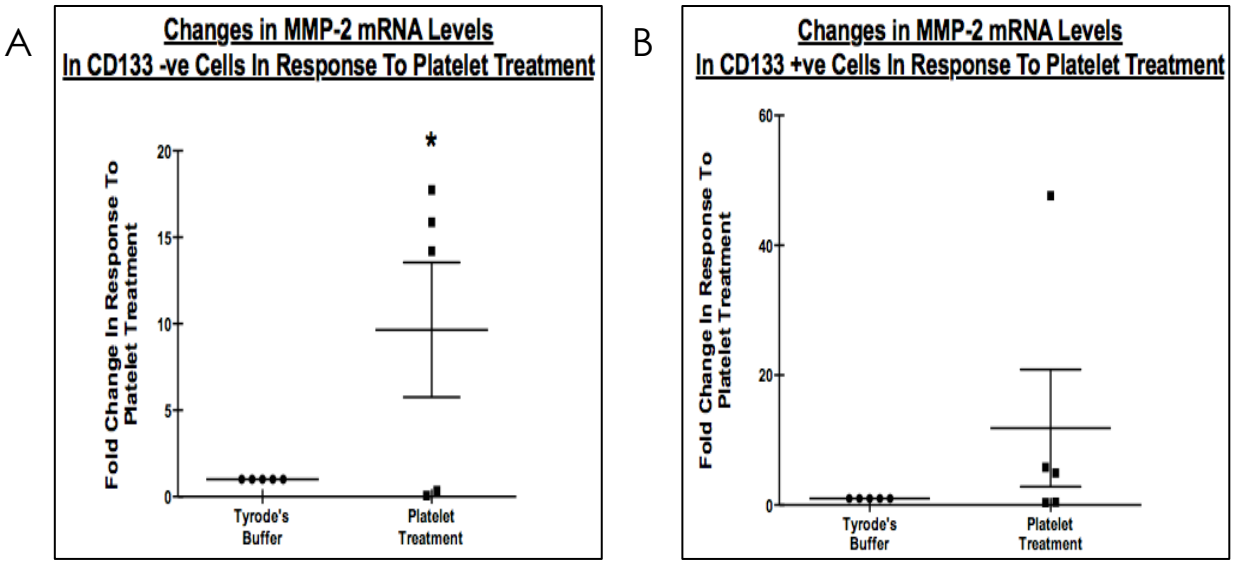


Figure 26: MMP-2 mRNA expression in A549 cells. Summary scatter plot of MMP-2 mRNA expression in A. CD133-negative and B. CD133-positive A549 cells before and after 24-hour platelet treatment (A. $n = 5$, $p = 0.15$; B. $n = 5$, $p < 0.05$).

Discussion

The first report describing the participation of platelet in malignancy was published in 1865 (Trousseau, 1865). This phenomenon, named Trousseau's syndrome after the physician who first described it, originally described unexpected migratory thrombotic events, which were thought to be predictive of malignancy. The description of Trousseau's syndrome was later expanded to include other forms of thrombosis, including disseminated intravascular coagulation, migratory thrombophlebitis and pulmonary embolism (Gay & Felding-Habermann, 2011). Today, Trousseau's syndrome is used to describe any coagulatory/thrombotic episode in patients with malignancy (Varki, 2007). Compared to healthy individuals, cancer patients are known to have increased occurrence of thrombotic events as well as hyperactive platelets (Jurasz et al., 2004; Jurasz, North, Venner, & Radomski, 2003). Interestingly, the incidences of thrombosis in cancer patients are even higher in patients with metastatic cancers (Khorana & Fine, 2004). The importance of the involvement of circulating platelets in cancer development first became known from animal studies, where decreases in platelet count lead to corresponding decreases in cancer metastasis in mice (G. Gasic & Gasic, 1962; G. J. Gasic et al., 1968). Thereafter, many proteins secreted by platelets such as VEGF, PDGF, ANGPT-1, SDF-1α and MMP-2 have been found to stimulate tumor angiogenesis and cancer metastasis (Holopainen et al., 2009; J.-K. Li et al., 2008; Mendes, Kim, Lungu, & Stoica, 2007; Pietras, Sjoblom, Rubin, Heldin, & Ostman, 2003; Verheul et al., 2000). Animal models were used as well to show the involvement of platelet receptors such as GPIbα and GPVI in platelet-supported metastasis (Jain, Russell, & Ware, 2009; Jain et al., 2007). Furthermore, platelets can also promote metastasis via interaction with cancer cells in a process known as TCIPA. During TCIPA, platelets promote the invasion, metastasis and extravasation of cancer cells through both direct physical interactions with the cancer cells as

well as the release of chemokines and growth factors to promote cancer cell growth and survival in a paracrine manner (Jurasz et al., 2004).

Cancer cells utilize the lymphatic system and the vascular system in their metastasis to distant sites. Platelets, as a major component of the vascular system and via the secretion reaction at sites of leaky tumor vessels can communicate with other cells such as endothelial cells and immune cells in the tumor microenvironment to facilitate cancer metastasis (Yan & Jurasz, 2016). One popular theory of a possible mechanism behind cancer metastasis is the seed and soil hypothesis. This theory suggest that metastasis will only form when cancer cells with certain characteristics (the "seeds") are planted in specific microenvironments (the "soil") that are suitable for growth (Paget, 1989). This theory was later refined to suggest that both the anatomy of the circulatory route as well as tumor microenvironment characteristic are important for the metastasis (Fidler, 2003; Langley & Fidler, 2011). One type of cell that fits the characteristics of the "seed" is the CSC. CSCs consists of a small population of highly invasive cancer cells with stem cell-like characteristics (Kreso & Dick, 2014). The defining characteristics of the CSCs is their ability to self-renew by producing two daughter cells, one of which is identical to the parent cell that is able to retain the unlimited capacity to replicate. The other daughter cell will have progenitor cell characteristics, and can give rise to differentiated cells that makes up the bulk of the tumor (Reya et al., 2001). Thus, CSCs are thought to drive cancer metastasis, initiate new tumors, and maintains the tumor bulk (Lobo et al., 2007). Indeed, the existence of CSCs, as identified by the expression of CSC markers, has been linked to increased rates of metastasis and worse prognosis in patients with brain, bone, breast, colorectal, and lung

cancers (Cherciu et al., 2014; Ohi et al., 2011; Sholl et al., 2010; L. Wang et al., 2011; Zeppernick et al., 2008).

Since platelets have been shown to participate in cancer metastasis, it is reasonable to speculate that platelet-supported metastasis may partially be due to platelets' effect on CSCs. One factor that has been found to aid in CSC metastasis and is also found in platelets is SDF-1α. SDF-1α is a chemokine that is physiologically involved in the retention of hematopoietic stem cells within the bone marrow through interaction with their receptor CXCR-4 (Stellos & Gawaz, 2007). Under circumstances where malignancies are present, the SDF-1α: CXCR-4 signalling pathway is associated with the proliferation and invasion by ovarian and pancreatic cancer cells (Q. Guo et al., 2014; Luker et al., 2012; Shakir et al., 2015). SDF-1α and CXCR-4 interaction was also found to be associated with the proliferation and chemotherapy resistance of both primary and metastatic breast cancer (B. C. Lee, Lee, Avraham, & Avraham, 2004; Sison, McIntyre, Magoon, & Brown, 2013; Smith et al., 2004). Furthermore the SDF-1α: CXCR-4 signalling pathway can also contribute to the intravasation of cancer cells into the lymphatic system (Hirakawa et al., 2009).

SDF-1α: CXCR-4 is an important signalling pathway for the motility of CSCs (Lapidot & Kollet, 2002; Massberg et al., 2006; Smith et al., 2004). The of SDF-1α on cancer cell motility and invasion is partially dependent on MMPs. Specifically, MMP-2 and MMP-9 expression is up-regulated by SDF-1α and CXCR-4 interaction (Pan et al., 2013; Tang et al., 2008). Moreover, SDF-1α was found to be able to stimulate platelet activation (Karim et al., 2016). SDF-1α mediated platelet activation can lead to further release of SDF-1α from platelet granules (Chatterjee & Gawaz, 2013; Massberg et al., 2006). Thus, it is possible that SDF-1α stimulate

cancer cell metastasis through platelet activation. Considering that platelets are a major source of SDF-1α in the vasculature, I hypothesized that platelet-derived SDF-1α can preferentially stimulate the invasion of CSCs through interaction with CXCR-4 expressed on CSC surfaces. Moreover, this preferential stimulation in invasion is dependent on the up-regulation of MMP-2 and MMP-9 by CSCs. To confirm platelets release SDF-1α upon stimulation, the concentration of SDF-1α in collagen-aggregated human platelet releasates was compared to that of resting platelet releasates. The result agreed with previous reports that SDF-1α is actively secreted by human platelets (Chatterjee & Gawaz, 2013).

Although serial transplant remains the only method for the conformation of self-renewal and tumor initiating abilities of suspected CSC populations, the characterization of CSC markers had greatly simplified the process of CSC identification (Al-Hajj et al., 2003; Eramo et al., 2008; Lapidot et al., 1994; O'Brien et al., 2007; Singh et al., 2004; Z. F. Yang et al., 2008). Common markers that have been used to identify CSCs include CD44, CD24, ALDH-1 and CD133 (Visvader & Lindeman, 2008) (Table 3). Moreover, Hoechst 33342 dye excretion, a method that has been used for the isolation of hematopoietic stem cells, was later found to be also useful for CSCs (Goodell, 2005; Goodell et al., 1996; Goodell et al., 1997). Similar to hematopoietic stem cells, the Hoechst-negative SP cells that were identified based on dye excretion was shown to be a result of ABC transporter overexpression. Moreover, the SP cells possessed characteristics of CSCs, including asymmetrical division, and the ability to sustain colonies (Hirschmann-Jax et al., 2004). Thus, Hoechst 33342-negative SP was utilized as one of the means of CSC identification in my experiments.

The human lung carcinoma cell line A549 has been used in a number of studies investigating the role of platelets in metastasis (Gong, Mi, Zhu, Zhou, & Yang, 2012; Janowska-Wieczorek et al., 2005; Jurasz et al., 2001; Y. Li, Miao, Xiao, Cai, & Zhang, 2014). Importantly, a Hoechst-negative SP with CSC-like characteristics such as asymmetrical differentiation and colony formation were identified in the A549 cell line (Q. Guo et al., 2014; Xie et al., 2011). Following the Goodell protocol, Hoechst 33342 (5μM) and Verapamil (50μM) was used to confirm the presence of SP in A549 cells. The results agreed that a Hoechst-negative SP exist in the A549 cell line.

The effect of platelet releasates on total and SP A549 cell invasion were studied in gelatin-coated Boyden chambers-like assays. In these experiments, the gelatin-coated membrane of the chambers was used to simulate the ECM, while platelet releasates were used because SDF-1α is secreted from platelet granules upon platelet aggregation. The result show that compared to resting platelet releasates, collagen-aggregated platelet releasates preferentially stimulated the invasion of A549 SP cells. This suggested that the aggregated-platelet releasates may contain factors that can promote the invasion by SP cells. Next, the inhibitors AMD3100 and GM6001 were used to determine whether the preferential stimulation by SP cells by collagen-aggregated platelet releasates is dependent on the SDF-1α: CXCR-4: MMP pathway. AMD3100, also known as Plerixafor, is a small non-peptide antagonist of the receptor CXCR-4, whereas GM6001 is a broad spectrum MMP inhibitor (Awasthi, Wang-Su, & Wagner, 2008; Cashen, Nervi, & DiPersio, 2007). Neither AMD3100 nor GM6001 was able to decrease the invasion of Hoechst-negative SP-identified CSC in response to collagen-aggregated platelet releasates. However, AMD3100 was able to decrease total A549 cell invasion in these experiments.

Although non-significant, GM6001 also showed a trend of decrease in total A549 cell invasion. This data supports previous reports of AMD3100 being able to decrease metastasis of colorectal cancer in vitro and head and neck cancers in mouse models (J.-K. Li et al., 2008; Yoon et al., 2007). However, it conflicts the idea that metastasis is driven by SP-identified CSCs. A possible explanation could be that since the SP express higher levels of ABCG2 drug transporters, they are able to excrete the AMD3100, GM6001 as well as the Hoechst dye. Moreover, it is possible that the Hoechst-negative SP may not an accurate representation of CSCs. Tumors contains cells that are highly heterogenic and many of these cells are known to be chemotherapy resistant by expressing higher levels of ABCG2 drug transporter. As a result, the Hoechst negative SP might not only contain cells with stem cell like characteristics, but also cells that are chemo-resistant (You, Morris, & Wang, 2007). For the A549 cell line alone, SP percentage has been reported to be anywhere between less then 1% to 24%, with most also reporting that stem-cell like properties are possessed by these SP cells (Ho et al., 2007; Sung et al., 2008; Xie et al., 2011; Y. Yang et al., 2015). Thus, it is difficult to determine how much of SP cells actually possess stem cell-like characteristics.

Depending on the method of detection the percentage of CSCs vary largely (Ho et al., 2007; Sung et al., 2008; Xie et al., 2011; Y. Yang et al., 2015). This perhaps is an indication that the Hoechst negative SP may not be a specific indicator of CSCs. Especially because many cells within a tumor would also have higher expression levels of drug transporters that may contribute to their drug resistance ability (You et al., 2007). Thus, CD133, a CSC marker that has been extensively used for the identification of CSCs in many tumor types including prostate, liver, lung and skin cancers, was used in an attempt to validate Hoechst-negative SP-identified cells as

CSCs (Kubo et al., 2013; Ma et al., 2007; Monzani et al., 2007; Shepherd et al., 2008). CD133, also known as prominin-1, is a transmembrane glycoprotein (Miraglia et al., 1997). The expression of CD133 on A549 cells has been previously reported, and was confirmed in this study (Roudi et al., 2014; H. Zhang et al., 2014; H. Z. Zhang et al., 2010). Moreover, investigation of CD133 surface expression by the Hoechst-negative SP indicated that the SP is enriched with CD133-positive cells, although CD133-positive cells still represent a minority of SP cells.

Gelatin-coated Boyden chambers were used to investigate the effect of collagen-aggregated platelet releasates on the invasion of CD133-positive and CD133-negative A549 cells. Neither AMD3100 or GM6001 caused a decrease in the total number of A549 cells that have invaded, contrary to experiments using Hoechst-negative SP. However, the general trend in both sets of experiments was similar. Since experiments using CD133-identified CSCs only involved three individual experiments, whereas SP-identified CSC experiments had a N number of 13, it is likely that further experiments with CD133-stained cells are required to increase the power of the statistical test.

Neither AMD3100 or GM6001 induced a decrease in SP invasion. However, both inhibitors induced a decrease in CD133-positive cell invasion in response to collagen-aggregated CSCs. Although the A549 SP is enriched with CD133-expressing cells, still only a very small population of A549 cells are positive for CD133 surface expression. Thus, it is possible that any effect of AMD310 and GM6001 on CD133-positive cells in the SP were masked by the much

more abundant CD133-neagtive cells. As a result, no inhibition of invasion would be seen in total A549 cell invasion.

The concept of tumor plasticity has gained much attention within the recent years. Tumor plasticity means that a tumor is capable of transforming itself to a phenotype that is beneficial for its survival (Cabrera, Hollingsworth, & Hurt, 2015). There have been reports of non-CSCs gaining stem cell-like properties and are able to switch between "CSC-like" and non-CSC state in response to different environmental stimuli (Chaffer et al., 2011; Chaffer et al., 2013; Roesch et al., 2010). This change between "CSC-like" and non-CSC state is known as CSC plasticity (Cabrera et al., 2015). The process of EMT has been implicated in playing a role in CSC plasticity where studies have showed the activation of EMT-associated pathway, such as Ras activation, induced stem cell-like traits in cancer cells including growth advantage and tumor generating abilities (Mani et al., 2008; Morel et al., 2008). Physiologically, EMT is an important process that occurs during early development and tissue injury because it allows for the transformation of epithelial cells into progenitor cells with mesenchymal phenotypes, which contribute to the formation of secondary epithelia through progenitor cell differentiation (Kalluri & Weinberg, 2009). Signaling pathways such as TGFβ and Ras as well as transcription factors such as Snail and Slug, that are known to participate in physiological EMT, are also active during cancer development (Son & Moon, 2010; Thiery, 2003). In fact, some argue that there are no real CSCs, but rather normal cancer cells undergoing EMT and gaining stem cell-like characteristics (Gupta et al., 2009). Since platelets are known to induce EMT in some cancer cells, it could be argued that the preferential effect of platelet releasates on CSC invasion is a result of platelet-induced EMT in these cancer cells (Labelle et al., 2011). To investigate this

possibility, the effect of platelet contact on A549 cell CD133 surface expression was tested. Incubation of A549 cells with either platelet releasates or washed platelets did not affect the level of CD133 surface expression by A549 cells, thus indicating that the preferential stimulation of CD133-identified CSC invasion by collagen-aggregated platelet releasates was not due to platelet induced EMT program that may have caused the conversion of CD133-neagtive cells into CD133-positive cells.

SDF-1α: CXCR4-induced cellular migration and cancer cell invasion is, at least in part, dependent on up-regulation of MMP-2 and MMP-9 expression, which can lead to basement membrane and ECM degradation (F. Jin et al., 2008; Osman & Osman, 2016; Pan et al., 2013). Moreover, the greater overall invasion by CD133-positive A549 cells and the decrease in CD133-positive cell invasion by GM6001 in platelet-stimulated CSC invasion assay suggested a possible role of MMP-2 and -9 in platelet-stimulated CSC invasion. Gelatin zymography was used to compare MMP-2 and MMP-9 levels in CD133-positive and CD133-neagtive A549 cells. CD133-positve cells had higher basal levels of MMP-2 protein. However, the addition of platelets to the cancer cells increased the MMP-2 levels in CD133-negative cells. To account for the possibility that the increase in MMP-2 originated from platelet granules, qPCR was used to investigate the mRNA expression levels of MMP-2 in CD133-positive and CD133-negative A549 cells. qPCR revealed that platelets caused a significant increase in MMP-2 mRNA expression levels after 24-hour incubation with washed human platelets, indicating that the increase in MMP-2 protein in CD133-negative cells incubated with platelets was a result of increased transcription expression of MMP-2 by the cancer cells. Further, it is likely that CD133-positive cells showed higher invasion in response to both resting and collagen-aggregated

platelet releasates due to having higher basal levels of MMP-2. MMP-9 levels were minimal in both CD133-positive and CD133-negative populations and were not significantly affected by co-incubation with platelets. These results are in agreement with that of Guo et al whom have suggested that MMP-2 would be a better predictor of metastasis then MMP-9 in lung cancer (C. B. Guo et al., 2007). Contrarily, an increase in pro- and active MMP-9 was previously detected in breast cancer cells after 24 hours of co-incubation with platelets (Alonso-Escolano et al., 2006), suggesting that different forms of cancer may utilize different MMPs during platelet-stimulated invasion.

Although the invasion assays using the AMD3100 and GM6001 suggested that the invasion of CD133-positive cells was dependent on SDF-1α: CXCR-4 signalling and MMP-2, these inhibitors did not significantly affect CD133-negative cell invasion in invasion assays with CD133-stained A549 cells. However, further experiments are needed since the CXCR-4 inhibitor AMD3100 caused inhibition of total A549 cell invasion, which consists of mostly CD133-negative cells. These results suggest that perhaps other platelet factors may be behind the increase of MMP-2 protein in CD133-negative cells or perhaps that SDF-1α may have different influence on CD133-positive and CD133-negative A549 cells. Moreover, longer invasion assay durations may be needed to see the effect of increased MMP-2 in the CD133-negative cells.

Concluding Remarks & Future Directions

1. Concluding Remarks

Platelets are a major source of SDF-1α in the vasculature. Since SDF-1α: CXCR-4: MMP is a well-known signalling pathway for the mobilization of stem and cancer stem cells, and that cancer stem cells are thought to drive metastasis and initiate tumors, it is reasonable to hypothesize that platelet derived SDF-1α will have a preferential effect on the invasion of CSCs over the rest of the tumor cell population. In summary, the result of my study suggest that:

1. SDF-1α is confirmed to be actively secreted from human platelets.

2. The Hoechst-negative SP is enriched with CD133 expressing A549 cells.

3. Collagen-aggregated platelet releasates preferentially stimulated the invasion of Hoechst 33342-negative side population-identified as well as CD133 surface expression-identified CSC in the A549 cell line.

4. Co-incubation of A549 cells with platelets do not affect the expression of CD133 by A549 cells. The preferential stimulation of the collagen-aggregated platelet releasates on the invasion of CD133-positive A549 cells was an effect of platelet releasates acting on true CD133 expressing cells and not CD133-negative cells that may have converted into CD133-positive cells.

5. CD133-positive cells express higher basal levels of MMP-2. However, platelet-cancer cell interaction increases MMP-2 expression in CD133-negative, but not CD133-positive A549 cells.

6. SDF-1α and CXCR-4 interactions are likely behind platelet-stimulated invasion of CD133-positive A549 cells. It is likely that the SDF-1α and CXCR-4 interactions may also affect CD133-negative A549 cell invasion. However, further experiments are needed.

2. Future Directions

Releasates from collagen-aggregated platelets preferentially stimulated the invasion of both Hoechst-negative SP and CD133-positive A549 cell populations. AMD3100 and GM6001 were able to decrease this preferential invasion of CD133-positive cells induced by platelet releasates. The results imply that the invasion of CD133-positive cells may be dependent on both SDF-1α signalling and cancer cell MMP expression. To examine whether the same pathways are involved in platelet-induced A549 SP invasion, invasion assay in the presence of AMD3100 and GM6001 in response to collagen-aggregated platelet releasates may be performed after treatment with the ABCG2 inhibitor Verapamil. Since the overexpression of ABCG2 in SP cells contributed to the excretion of Hoechst, it is likely that they also contributed to the excretion of AMD3100 and GM6001 during the invasion assay.

CD133-positive cells have higher basal levels of MMP-2. However, co-incubation of A549 cells with platelets only increased the mRNA expression of MMP-2 in CD133-negative cells. Moreover, even though there was an increase in CD133-negative cell MMP-2 mRNA levels, the addition of AMD3100 did not affect isolated CD133-negative cell subpopulation invasion in response to collagen-aggregated platelet releasates. However, AMD3100 did inhibit total A549 cell invasion in Hoechst-stained invasions. These results suggest that there might be differential pathways regulating the invasion of CD133-positive and CD133-negative A549 cell invasion. However, it is also possible that the low number of experiments (n = 3) resulted in the experiments being underpowered and thus more experiments need to be performed. Moreover, the difference between CD133-positive and CD133-neagitve A549 subpopulations should be

investigated. For example, the expression of CXCR-4 on CD133-positive in comparison to CD133-negative A549 cells should be investigated. Differential expression of CXCR-4 could contribute to the ineffectiveness of AMD3100 in CD133-negative cells. The platelet aggregating ability, the self-renewal and tumor initiating ability of both populations using serial transplantation can also be investigated. Lastly, whether the current observations extend to other cell line of lung cancer and different types of cancer may be studied.

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