THE ROLE OF MICROVESICLELS IN THE HYPERCOAGULOPATHY ASSOCIATED WITH PROSTATE CANCER

THE ROLE OF MICROVESICLELS IN THE HYPERCOAGULOPATHY ASSOCIATED WITH PROSTATE CANCER

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ABSTRACT

Patients with prostate cancer (PC) are at high risk of developing migratory thrombosis compared to healthy individuals. This is due to the haemostatic abnormality as a result of the presence of cancer, and is referred to as Trousseau's syndrome. Trousseau's syndrome leads to increased mortality among cancer patients, and is considered the second cause of death after cancer itself. We investigated the role of microvesicles (MVs), which are circular membrane compartments shed from cancer as well as from healthy cells, in the development of Trousseau's syndrome. We compared the pro-coagulant activities between MVs derived from PC cell lines with different oncogenic and metastatic characteristics, using chromogenic assays to determine their thrombin generation. Microvesicles from the more aggressive DU145vIII and more metastatic PC3-MLN4 show increased thrombin generation compared to MVs derived from DU145 and PC3. We also compared thrombin generation in MVs extracted from plasma of PC patients of various cancer stages. MVs from PC patients with a metastasized tumour had increased thrombin generation compared to patients with localized tumours. Finally, we transfected the CHO cell line with the human protease-activated receptor 1 (hPAR1), the principal receptor of thrombin. PC MVs led to the activation of PAR1 in CHO (hPAR1), indicating thrombin generation. Our in vitro studies suggest a potential role of PC MVs in the migratory thrombosis observed in Trousseau's syndrome, due to their independent ability to generate active thrombin. We also demonstrated that thrombin generation of PC-derived MVs correlated with the oncogenic and metastatic characteristics of prostate cancer.

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CHAPTER ONE: INTRODUCTION

1.1 Prostate Cancer

Incidence and mortality

Prostate cancer is the third leading cause of cancer among men of North America, falling under colorectal and lung cancer (American Cancer Society, n.d., (Canadian Cancer Statistics, 2016). In Canada, it is considered to be the most commonly diagnosed cancer among men, and the fourth among all cancers with lung, colorectal, and breast cancers leading the charts. Prostate cancer accounts for an estimated 21,600 new cases in 2016. That is 21% of all cancers in men, and 11% of all cancers in Canada (Canadian Cancer Statistics, 2016). Over half of all cancer incidence rates for those over the age of 50 in Canada are attributed to the four most significant ones, those are the colorectal, breast, lung and prostate cancers (Canadian Cancer Statistics, 2016). In general, incidence rates have been declining steadily since the start of the century, after peaking in 2001 with the wide-spread use of the Prostate Specific Antigen (PSA) screening test.

The burden of prostate cancer in Canada is significant among men. With an estimated 4,000 death from Prostate cancer in 2016, it accounts for 10% of all cancer deaths in Canadian men. Prostate cancer sits among the five most lethal cancers in Canada among lung and bronchus, colorectal, breast, pancreatic, and prostate cancers, with lung cancer amounting to an estimated high of 21,800 deaths in 2016 alone (Canadian Cancer Statistics, 2016). It is estimated that the probability of dying from prostate cancer over the next decade in Canada is almost negligible below the age of 60,

but rises to 0.3% over the age of 60 and is increased 10-fold when one reaches 80 years of age (Canadian Cancer Statistics, 2016). Mortality of prostate cancer is found among the four most deadly types of cancer in men in Canada, being pancreatic, colorectal and lung cancer with rates of 0.3%, 0.6% and 1.8% respectively at the age of 60 (Canadian Cancer Statistics, 2016). Therefore mortality is also significantly associated with age. Luckily, with prostate cancer being the most common cancer but having the third highest mortality rate among men, it indicates the increased likelihood of surviving and reflects the successful early detection and treatment methods.

Risk Factors

There are numerous risk factors putting individuals at risk of prostate cancer, with the most common ones being family history and age. To represent the effect of heredity, men who have an immediate family member with diagnosed prostate cancer have about 2 to 3 times the risk of developing prostate cancer compared to those who don't (Hsieh et al., 2003). In the general population, individuals with a hereditary link among the family have an incidence rate of about twice 15% for prostate cancer, compared to 3% in those without a family history (Hsieh et al., 2003). The other most predictive risk factor is age, which indicates that older individuals are at a higher risk of developing prostate cancer compared to younger individuals. Particularly, men above the age of 65 years, experience an 18% increased risk compared to younger men in Canada (Canadian Cancer Statistics, 2016). Furthermore, Cancer Statistics Canada report the lifetime probability of developing prostate cancer in 2010 to be less than 0.05% below the age of 40, which

dramatically increases to 0.2% and 1.6% at the ages of 40 and 50, therefore showing a

non-linear trend of increased risk as one ages (Canadian Cancer Statistics, 2016).

Metastasis

Prostate cancer is associated with many metastatic sites, most commonly in the

bone, lymph nodes and the liver (Gandaglia et al., 2014). Nearly 80% of patients with

advanced prostate cancer present with bone metastasis, while 10% of patients have a

metastatic involvement of lymph nodes and the liver (Gandaglia et al., 2014). It is not

uncommon for patients to acquire multiple metastatic sites, and those individuals amount

to about 18% of the prostate cancer population in the United States (Grandaglia et al.,

2014).

1.2 Haemostatic Dysregulation in Cancer

With such a high incidence rate of prostate cancer, comes a significant burden on

healthcare resources, with increased screening and chemotherapy costs (Cohoon et al.,

2015). Among the many complications of prostate cancer, such as incontinence, erectile

dysfunction and dysuria, haemostatic dysregulation is considered a significant

consequence with an associated mortality (Michaelson et al., 2008 & Heit et al., 2016).

However, haemostatic dysregulation is nevertheless present in all types of cancer,

including prostate cancer, breast, colorectal, pancreatic and lung cancer (Chew et al.,

2006). It is well known that cancer alters the state of blood in patients, resulting in a

condition that renders the blood prone to spontaneous coagulation events (Cheang et al.,

2004). In the clinic, cancer patients show abnormal coagulation tests, such as an

imbalance of coagulation proteins and significantly reduced times in laboratory clotting tests (Edwards et al., 1987).

A French internist, Armand Trousseau, first observed cancer-related hyper coagulopathy. Armand discovered that patients who presented with unexplained and idiopathic thrombotic events later manifested a visceral malignancy (Trousseau A., 1865). The syndrome comes with a significant cause of mortality among cancer patients. In fact, Trousseau's syndrome represents the second cause of death after cancer itself (Khorana et al., 2007). In general, Trousseau's syndrome is a term given to any state of hemostatic alteration or hyper-coagulopathy in association with hyperplasia, therefore Trousseau's syndrome is unique to cancer patients (Varki A., 2007). Up to 20% of cancer patients receive idiopathic thrombotic complications as part of Trousseau's syndrome (Heit et al., 2016).

The most common clinical observation of Trousseau's syndrome is migratory venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE). Deep vein thrombosis occurs through blood stasis in the major veins of the lower limbs, which upon dislodging, the thrombus may travel through the circulation, where it's called a thrombi. A thrombi may travel through the heart and to the lungs blocking a pulmonary artery. This forms a pulmonary embolism, which depending on the size and location may be fatal. Cancer acts as an independent risk factor for migratory VTE, resulting in 4-6 times as much increased risk compared to healthy individuals (Walker et al., 2013). In addition, hyper-coagulation in cancer patients includes a wide spectrum of thrombotic events, such as disseminated intravascular

``coagulation, platelet-rich microthrombi, and verrucous endocarditis among many other thrombotic disorders (Varki A., 2007, Sallah et al., 2001). Importantly, patients with Trousseau's syndrome are often asymptomatic; post-mortem studies of patients with advanced cancer showed an increasingly underdiagnosed VTE in cancer patients (Johnson et al., 1999). Thus, idiopathic venous thromboembolism may act as the first sign of an underlying malignancy, and can present a significant risk of damage to distant vital organs such as the heart, lungs and brain.

Trousseau's syndrome and its associated VTE is also present in the pediatric population. Although very little studies investigate the risk of VTE in pediatric cancer patients, a few report a similar association between cancer and the increased risk of VTE (Massicotte et al., 1998, Journeycake et al., 2006). In a Canadian registry of children receiving central venous lines and diagnosed with venous thromboembolism, more than 40% of children also had cancer, accounting for the highest proportion of CVL-related deep vein thrombosis (Massicotte et al., 1998). Similarly in the adult cancer population, asymptomatic VTE is common among children with cancer, and is associated with increasing mortality, further suggesting the need for understanding the role of cancer in the development of asymptomatic venous thromboembolism (Journeycake et al., 2006, Halton et al., 2012).

Patient and tumour-related risk factors for VTE

The incidence of VTE varies among cancer patients, and this is due to the differences of risk factors among patients. Most of the related risk factors are tumour

related, but there are also patient and treatment-related risk factors for VTE. For instance, among the tumour related risk factors, increased tumour grade and stage correlate greatly with the incidence of VTE. Epidemiological studies report that, in general, the more advanced the cancer stage, the higher risk the risk of VTE development in a patient (Blom et al 2005, Chew et al 2006). For instance, the rate of VTE is increased 3-fold in the presence of metastasized tumours compared to localized tumours in cancer patients (Dickmann et al 2013).

In patient-related risk factors, the state of patients is a significant factor to consider in the development of VTE. Comorbidity with existent conditions, for example, is a major factor when considering cancer-associated VTE. Diseases such as obesity, systemic infection, and prolonged hospitalization due to various reasons have been linked to VTE in cancer patients (Khorana et al., 2009). On the other hand, previous history of VTE in the patient's family has been investigated to show certain mutations that aid in the development of VTE in cancer patients (Gran et al., 2016).

Treatment related risk factors for VTE include chemotherapy drugs and surgery. Receiving chemotherapeutic drugs is considered a significant and independent risk factor for VTE development (Khorana et al., 2005). Various chemotherapeutic drugs are widely known to be involved with cancer-associated thrombosis. Bevacizumab, an anti-angiogenic drug used in chemotherapy was shown to be significantly associated with the incidence of VTE (Nalluri et al., 2008). In addition, in a large retrospective chart review of more than 1,900 cancer patients, those who received platin-based chemotherapeutic drugs were three times more likely to develop venous thromboembolism than cancer

patients receiving other drug compounds (Di Nisio et al., 2010). In general, various studies report a significant link between chemotherapy treatment and VTE in cancer, where it is reported that the risk of VTE is increased by about 2 to 6-fold in patients undergoing chemotherapy compared to cancer patients who aren't (Heit et al., 2000, Blom et al., 2006).

Pathophysiology of cancer-associated thrombosis

The main initiator of the extrinsic pathway of coagulation is tissue factor (TF). Tissue factor is mainly found beneath the vascular endothelium, which upon vessel injury, is exposed to components of the circulation which starts the clotting cascade. Serum Factor VII is activated and forms the TF-FVIIa complex with TF, completing a chain reaction which leads to the formation of insoluble fibrin strands and a thrombus. Thus, exposure of TF is a key step in clot formation. Research on the link between cancer and thrombosis has mainly focused on the up-regulation of procoagulant molecules in tumour cells, specifically tissue factor (Mueller et al., 1992 & Gerotziafas et al., 2012). The up-regulation of TF correlates with tumour stage, aggressiveness and reduced survival rates among cancer patients (Kakkar et al., 1995, Ueno et al., 2000, Vrana et al., 1996). In a unique case, a male in his 50's presented with Trousseau's syndrome associated with lung carcinoma. Plasma levels of TF were 41 times as much as the average concentration in the plasma of 16 healthy individuals (Del Conde et al., 2007). Increased TF expression was even observed in the vasculature supplying the tumour (Del Conde et al., 2007).

Furthermore, another molecule, the Cancer Procoagulant (CP) has been studied in relation to Trousseau's syndrome. Cancer procoagulant is understood to be a cysteine proteinase, a polypeptide composed of 674 amino acids with no carbohydrate component (Gordon and Mourad, 1991, Falanga et al., 1985). What is remarkable about CP is that it directly on Factor X, resulting in its cleavage and activation to FXa, and this occurs independently of FVa (Gordon and Mourad, 1991, Mielicki and Gordon, 1993). Cancer Procoagulant is expressed in malignant cells and fetal placental cells, and exhibit increased pro-coagulant activity (Gordon and Benson, 1989). Interestingly, CP is highly expressed in malignant tumour tissue, but not in benign tumour tissue (Donati et al., 1985). Not only is CP overly expressed within cancer cells, but its levels in the blood are elevated in cancer patients compared to healthy individuals (Gordon and Benson 1989).

On the other hand, thrombus formation is the result of two pathways, the coagulatory and fibrinolytic pathways. In cancer, those pathways exist in an imbalance, where the coagulatory pathway ending with thrombin production, is enhanced and over-activated while the fibrinolytic pathway which involves plasmin, is inhibited. Plasmin forms from plasminogen by the action of tissue-type plasminogen activator (t-PA) or urokinase plasminogen activator (uPA), however, plasmin formation is deactivated by the plasminogen activator inhibitor-1 (PAI-1). PAI-1 is observed in many cancers, and its expression and mRNA levels are overexpressed in various cancers such as ovarian, myelomas, colorectal and lung cancer (Yagci et al., 2003, Casslen et al., 1994, Pyke et al., 1991). In one study, levels of all uPA, its receptor uPAR and PAI-1 were required and correlated with the invasiveness of human lung cancer cells (Liu et al., 1995). This

evidence surrounding the overexpression of PAI-1 in tumour cells further indicate the dual role of cancer in haemostatic dysregulation.

1.3 Microvesicles

Microvesicles is a term falling under the all-encompassing extracellular vesicles (EVs). Extracellular vesicles are small membrane-shed vesicles that are released by both healthy and pathological cells, such as tumour cells. EVs can be referred to by several terms existent in the literature. Those include exosomes, microparticles, apoptotic bodies, oncosomes as well as ectosomes, and the main features differentiating them are size and method of release. The two major classifications are microvesicles (MVs) and exosomes, with microvesicles being larger in size (100-1000 nm) than exosomes (<50-100 nm) (Al-Nedawi, Meehan & Rak, 2009). Microvesicles, which often are also called ectosomes, form in a mechanism characterised by the direct budding of vesicles from the cellular surface and into the cell's microenvironment in a process referred to as vesiculation (Ratajczak, Wysoczynski, Hayek, Janowska-Wieczorek & Ratajczak, 2006). On the other hand, exosomes are first formed within an endosome, which then fuses with the cellular membrane allowing the release of exosomes into the extracellular space (Williams & Urbe, 2007; Trajkovic et al., 2008). Often, the term microvesicles is used to refer to a heterogeneous mixture of many types of EVs, and thus will be used in this report.

The most striking functional properties of MVs lies in their ability to act as cargo vesicles for transporting active molecular components. For instance, microvesicles can carry genetic material (DNA, mRNA, miRNA and siRNA), growth factors, hormones,

signalling molecules, as well as drugs during the efflux of chemotherapeutic agents from tumour cells (Colombo et al., 2014; Rak, 2013). Depending on the surroundings of a cell, MVs can be released into various bodily fluids, including the blood, urine, lymph, cerebrospinal fluid, mucus and saliva (Lee et al., 2011). Thus, combined with their comprehensive biological content, the release of MVs around cells enables them to participate in a wide spectrum of cell-to-cell communications mediating responses in local and distant targets. The interactions by which MVs exert their effects are complex, and they include direct surface contact with their target cells, release of factors into their surroundings, and most importantly the transfer of biological components into recipient cells (Hendrix, Westbroek, Bracke & DeWO, 2010).

In cancer, both the rate and the amount of microvesicle vesiculation in tumour cells are greatly increased. This phenomenon was observed to be related to cellular pathways that are characteristic of cancer development. For instance, oncogenes and the dysfunction of tumour suppressor genes were both linked to increased MVs release. Oncogenes such as the constitutively active EGFRvIII and K-Ras oncogene were shown to substantially promote microvesicle budding (Al-Nedawi et al., 2008; Yu et al, 2005). When MVs contain oncogenic material, they are referred to as oncosomes. Oncosomes have been linked to promoting tumour progression, angiogenesis as well as metastasis (Rak, 2013). As a general function of MVs, oncosomes can exert an impact on both tumour and other cells. For instance, oncosomes containing the oncogenic EGFRvIII induce growth and angiogenesis in recipient cells, promoting cancerous properties (Al-Nedawi et al., 2008). In addition, EGFRvIII-containing oncosomes may transform

endothelial cells to stimulate VEGF production, acting as a sign for increase in angiogenic characteristics. (Al-Nedawi K, Meehan B, Kerbel RS, Allison AC & Rak J, 2009). Moreover, the loss of certain tumour suppressor genes, such as the p53 gene, promotes increased vesiculation and impacts the content of cancer-derived MVs (Yu et al., 2005). Therefore, these various genetic alteration not only regulate cancer cell growth and progression, but also suggests genetic regulation of MVs production and function.

1.4 Microvesicles in cancer-associated Thrombosis

With the considerable evidence illustrating an increased expression of TF in cancer cells, little is known on the mechanism by which Trousseau's syndrome is manifested. Research on TF-bearing extracellular vesicles has provided a possible mechanistic explanation for the hemostatic abnormality as well as the occurrence of distant thrombotic events. The strength of such research stems from the fact that cancer-derived microvesicles are able to travel in the circulation, enabling them to exert various effects on key players of the coagulation cascade. Clinically, TF-bearing extracellular vesicles correlate with tumour presence in cancer patients (Zwicker et al., 2013). In one study, higher TF-positive microparticles in the circulation were associated with a lower survival rate in patients with breast and pancreatic cancers (Tesselaar et al., 2009). Interestingly, TF positive microparticles were independent of the host's TF in initiating a thrombotic reaction (Thomas et al., 2015). Indicating, their significant pro-coagulant role in thrombus formation.

In addition, in vitro and in vivo studies further illustrate the significance of procoagulant microvesicles derived from tumour cells. Cancer microvesicles are abundant in TF compared to microvesicles from normal cells, and correlate with increased procoagulant activity (Tiley et al., 2008). For instance, glioblastoma microvesicles reduced clotting time by more than half compared to normal plasma, signifying the strong capability to accelerate clotting time, which was inhibited upon co-incubation with anti-TF antibodies (Bastida et al., 1984). Moreover, exosomes from cancer cells exhibit properties of the fibrinolytic pathway (Al-Nedawi et al., 2005). Exosomes from mast cells are able to up-regulate the plasminogen-activator inhibitor (PAI-1) which inhibits tPA responsible for activating plasmin, the primary regulator of fibrinolysis (Al-Nedawi et al., 2005). Specifically, PAI-1 mRNA, protein expression, as well as promotor activity were all shown to be induced by mast cell exosomes, thus indicating a role in promoting clot formation (Al-Nedawi et al., 2005).

Mouse models have provided interesting findings about the relationship between pro-coagulant extracellular vesicles and the thrombotic phenomena (Thomas et al., 2015). In one study, an inferior vena cavae (IVC) stenosis model was prepared for which pancreatic tumour microvesicles were later injected into the circulation. Upon injection of cancer-derived MVs, mice showed an increase in coagulation makers in the blood, an enlarged thrombin size, as well as a significantly reduced survival rate, compared to the control mice (Geddings et al., 2016).

Collectively, cancer cell-derived MVs correlate with increased mortality among cancer patients, and express up-regulated levels of pro-coagulant molecules such as TF,

while demonstrating increased pro-coagulant activity, as demonstrated in coagulation tests.

1.5 The protease activated-receptors

As Trousseau's syndrome is characterized with the hyper-activation of coagulation, it is essential to understand the coagulation pathway in relation to cancer. The end product of the activation of coagulation is the protease enzyme, thrombin. In order for the coagulation cascade to elicit responses other than clot formation, the cascade exerts its effects on the vascular endothelium through thrombin mediated activation of the protease activated receptor. The protease activated-receptors (PARs) consist of 4 subtypes (PAR1, PAR2, PAR3 and PAR4), each activated by a different molecule, and all of which are categorized as G-protein coupled receptors. Once PARs are activated, their exodomain N-terminus is cleaved exposing a tethered ligand that is part of the receptor, which acts in an intramolecular fashion initiating down-stream signalling pathways. Activation of PARs include pathways involving normal cellular development and angiogenesis (Carmeliet 2001, Griffin 2001).

As cancer is characterised by abnormal cellular functions, such as uncontrolled cellular division and differentiation, angiogenesis and cell death, the PARs play a key role in cancer progression as they are involved in these cellular processes. The PARs have been implicated in many types of cancer, including breast, melanoma, colon and prostate cancers (Tellez et al., 2003, Chay et al., 2002, Darmoul et al., 2003 & Even-Ram et al., 1998). Not only are PARs over-expressed in tumours, but they also correlate with

the degree of tumour development and metastasis (Even-Ram 1998, D'Andrea 2001). This illustrates the important function of PARs in cancer progression.

The most studied protease activated receptor is PAR1. As PAR1 is the principal receptor of the protease enzyme thrombin, it is commonly referred to in the literature as the thrombin receptor (Coughlin S. R., 2000). Thrombin-mediated activation of PAR1 initiates downstream signalling pathways involved in cancer progression, such as promoting survival and angiogenesis, while altering apoptosis (Griffin et al., 2001 & Cannolly et al., 1996). Embryonic development studies for example, indicate a retarded angiogenesis due to PAR1 knockout (Griffin et al., 2001). Endothelial cells also require PAR1 to promote their survival (Cannolly et al., 1996). Moreover, thrombin's effects on PAR1 includes the regulation of apoptotic pathways, where it was shown to activate the Akt/PKB and altering caspase-3 and -9 levels (Salah et al., 2007). This indicates the crucial role of thrombin-mediated activation of PAR1 in angiogenesis and survival of tumour cells. Additionally, thrombin's actions on PAR1 enhances the metastatic properties of tumour cells. For instance, thrombin is involved in promoting motility and migration of melanoma cells through the activation of PAR1 (Shi et al., 2004). Thrombin's interaction with PAR1 was also shown to promote cellular adhesion of tumour cells towards the extracellular matrix through interactions with integrins (Even-Ram et al., 2001). Interestingly, warfarin treatment targeted towards thromboembolism reduced the incidence of recurrent cancer in patients who survived previous tumours (Schulman et al., 2000). Thus thrombin's activity in cancer is essential for the propagation of tumour growth, and is at least partly mediated through PAR1.

In prostate cancer, there is strong expression of PAR1, not only at tumour cells, but also throughout the vasculature of the tumour and at metastatic sites such as lymph nodes (Kaushal et al., 2006, Greenberg et al., 2003). In one study, PAR1 RNA levels and protein expression in a prostatic cancer cell line derived from a bone metastasis site (VCaP) had 2.5 times as much PAR1 RNA compared to a benign prostatic tumour cell line (DuCaP) (Chay et al., 2002). The metastatic properties of prostate cancer is thus significantly propagated through PAR1's downstream signalling. Greenberg and colleageus (2003) illustrate an important role of thrombin by activating the RhoA pathway in LNCaP cells through PAR1. This lead to significant changes in the cells' cytoskeleton and elongation, promoting invasion through the endothelial barrier (Greenberg et al. 2003). Furthermore, the ability of PAR1 to promote invasion and metastasis in prostate cancer is strongly mediated through production of matrix metalloproteinases (MMP's) (Wilson et al., 2004). Activation of PAR1 by an agonist increased MMP-2 by more than double compared to control levels (Wilson et al., 2004). As matrix metalloproteinase enzymes are correlated with cancer invasiveness and progression, their stimulation further suggests a key role for PAR1 in regulating cancerous properties in prostate cancer.

HYPOTHESIS

Based on studies reporting the elevation of prostate cancer-derived microvesicles in cancer patients, which is also associated with the incidence of migratory venous thromboembolism, we propose that prostate cancer-derived microvesicles have a significant pro-coagulant activity. We also propose that since mortality of venous thromboembolism is increased with the stage of cancer, the pro-coagulant activity of microvesicles is associated with the oncogenic properties and metastatic potential of prostate cancer.

OBJECTIVES

I. To assess the role of cellular oncogenic and metastatic characteristics in the thrombin generation by prostate cancer-derived microvesicles.

The pro-coagulant activity of prostate cancer-derived microvesicles will be assessed in terms of their thrombin generation. The role of the oncogenic EGFRvIII and highly metastatic prostate cancer cell lines will be assessed in their regulation of thrombin generation. In addition, microvesicles from prostate cancer patients with differing metastatic potentials of their tumours will also be investigated in terms of their thrombin generation.

II. To assess the role of thrombin generation on PAR1 activation.

We will assess the role of thrombin on the activation of PAR1, and how it impacts the cellular proliferation and survival pathways. In addition, prostate cancer-derived

microvesicles will be incubated with PAR1 to investigate their generation of functional

thrombin and the subsequent truncation and activation of PAR1.

CHAPTER TWO: MATERIALS AND METHODS

2.1 ANTIBODIES

The phospho-ERK 1/2, phospho-Alt, phospho-p38 and B-actin antibodies were purchased from Cell Signalling. The anti-PAR1 ATAP2 antibody was purchased from Sigma-Aldrich. All antibodies used were monoclonal. HRP-conjugated secondary antibodies were of rabbit and mouse origin, and purchased from Cell Signalling and Bio-Rad respectively.

2.2 CELL LINES

Cell lines have been purchased from ATCC (unless indicated below), and are grown in a recommended medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. The following cell lines were used:

DU 145 (ATCC HTB-81): Human epithelial prostate carcinoma, derived from a brain metastatic site. Grown in Glutamax Minimum Essential Media (MEM, Gibco), complete.

DU 145vIII: A variant of the DU 145 cell line but stably transfected with a constitutively active variant of EGFR (EGFRvIII), prepared as described previously (Al-Nedawi et al., 2008).

PC-3 (ATCC CRL-1435): A grade IV human epithelial prostate adenocarcinoma, derived from a bone metastatic site. Grown in Ham's F-12K (Kaighn's) Medium (Gibco), complete.

PC-3 MLN4: A more metastatic variant of the PC-3 prostatic cancer cell line (Glinsky, Glinskii, Stephenson, Hoffman, & Gerald, 2004).

CHO-K1 (ATCC CCL-61): Healthy ovarian epithelial cells from a Chinese hamster.

CHO (hPAR1): CHO-K1 cells transfected with a dually tagged-hPAR1 containing the mCherry and eYFP fluorescent tags, on the N-terminus and C-terminus, respectively.

2.3 COLLECTING MICROVESICLES FROM CELL CULTURES

Cell cultures were grown to 80% confluency in 12 T75 cell culture flasks. Existing medium was then replaced with serum-free media and incubated for 24 hours. The media was then collected and subjected to two successive centrifugations at 1,250 rcf for 5 min and 12,000 rcf for 20 min to remove cells and cell debris. Finally, microvesicles are pelleted at approximately 121,000 rcf (42,000 rpm) and re-suspended with 250 µL of PBS Saline Solution (Gibco). Microvesicles total protein was measured using a Bradford assay (Bio-Rad).

2.4 EXTRACTING MICROVESICLES FROM PATIENT PLASMA

Microvesicles were extracted from the plasma of prostate cancer patients in a similar manner as with cell cultures using high speed centrifugation. Plasma samples were diluted in a 1:1 ratio in PBS then subjected to 2 successive centrifugations for 30 min at 2000 rcf and 45 min at 12,000 rcf to remove cells and other cellular debris. This is followed by ultracentrifugation for 2 hours at approximately 109,000 rcf (42,000 rpm) to

pellet microvesicles which are then re-suspended in PBS in a similar fashion to collecting

MVs from cell cultures as described above.

2.5 IMMUNOBLOTTING

Cell samples were homogenized using RIPA lysis buffer, while microvesicle samples

using 4X sample buffer. RIPA lysis buffer contained 50 mM Tris (pH 7.4), 0.5% (wt/vol)

sodium deoxycholate, 1% (wt/vol) NP-40, 0.1% (wt/vol) sodium dodecyl sulfate (SDS)

and 150 mM sodium chloride. The 4X sample buffer contained 250 mM Tris (pH 6.8),

8% SDS and 40% glycerol. Samples were then diluted in a 1:1 ratio with 2X Leammli

Buffer (Bio-RAD) containing β-Mercaptoethanol, followed by heating for 10 min at

95°C. Samples were loaded in a 10% polyacrylamide gel containing Sodium dodecyl

sulfate (SDS), and resolved at 40V for 20min then at 120V for 1 hour. Using a wet

transfer apparatus, gels were transferred onto nitrocellulose membranes at 100V for 1

hour. Membranes were then stained using Ponceau S (Bio-Rad), blocked with 5% milk in

TBST buffer followed by overnight incubation with antibodies as indicated previously

(2.1 Antibodies). Enhanced chemiluminescence alongside X-Ray films were used to

develop membranes.

2.6 CELL TRANSFECTION

A plasmid construct of the human protease activated-receptor 1 (PAR1) with fluorescent

tags was provided as a gift from Dr Nabil Seidah (Kim et al., 2015). CHO cells were

cultured in F12K medium supplemented with 10% (vol/vol) Fetal Bovine Serum (FBS),

and 1% penicillin/streptomycin at 37 °C, 5% (vol/vol) CO2. CHO cells were seeded in a

6-well culture plate (100mm × 20mm) at a density of 5×10^5 Cells. For each well, 2 mL of serum-free F12K medium was mixed with 20 µL of Lipofectamine D293 reagent (SignaGen Laboratories), and 4 µL of the mcherry-hPAR1-eYFP dually tagged plasmid construct. Following 5 hours of incubation at 37°C, the medium was replaced with F12k media supplemented with 20% FBS and 1% penicillin/streptomycin.

2.7 THROMBIN PREPARATION

Bovine Thrombin was purchased from Sigma-Aldrich containing 10.8 mg of product in powder form. Thrombin activity is often reported in U/mL. According to the product manufacturer, thrombin powder contains 92 NIH units per mg of solid, which amounts to 1002.8 NIH (1 NIH unit ≈ 1.1-1.3 IU) per 10.8 mg. Thus, 1 U/mL thrombin amounts to approximately 0.01 mg of solid, and this was used to calculate the desired thrombin concentrations before treatment. Preparing 1 U/mL thrombin required 1.6 µL of thrombin per 1 mL of solution (medium/PBS), while 2 U/mL and 4 U/mL required 3.2 µL/mL and 64 µL/mL of thrombin respectively.

2.8 THROMBIN TREATMENT OF CULTURED CELL

After preparation of thrombin, cultured CHO (hPAR1) and were grown in 100mm × 20mm cell culture dishes (Corning) in supplemented media. Complete media was then replaced with serum-free media and incubated for 24 hours. Different thrombin concentrations were then incubated with either serum-free medium or PBS and incubated with cultured cells for 24 hours, after which cells were lysed with RIPA buffer and homogenized for analysis.

2.9 SPECTROPHOTOMETRY

Chromogenic Assay

The Chromozym TH thrombin substrate was utilized as a chromogenic assay for thrombin generation. Microvesicle samples of volume 50 µL were mixed with 150 µL of Tris-buffer (pH 8.3, 50 mM, 227 mM NaCl, and 1% BSA), 100 mM $CaCl_2$, and 0.3 mM Chromozym TH in a 96-well clear plate. The plate is incubated at 37°C and read continuously with a spectrophotometer at a wavelength 405 nm according to manufacturer instructions. Microvesicles from both cell cultures and patient plasma samples were used in the Chromozym TH chromogenic assay.

Detecting fluorescent tags

Following 24 hours of thrombin treatment (described previously in "Thrombin treatment of cultured cells"), the PBS supernatant was transferred into a black clear-bottom 96-well plate (Corning) and read under a fluorescence spectrophotometer for the detection of the fluorescent mCherry signal. Remaining cells in the culture well were washed with PBS and read under the spectrophotometer for the mCherry signal. An excitation wavelength of 575 nm and absorption of 610 nm were used to detect mCherry.

CHAPTER THREE: RESULTS

3.1 Expression of EGFRvIII in DU145vIII prostate carcinoma cells.

The oncogenic EGFRvIII is independent from ligand activation, as the ligand-binding site is missing, which renders the receptor's tyrosine kinase motif to be continuously phosphorylated and the receptor being constitutively active (Al-Nedawi et al., 2008). As EGFR is implicated in cellular proliferation in cancer, we investigated the role of the oncogenic EGFRvIII in relation to the pro-coagulant nature of tumour-derived microvesicles.

Figure 1A illustrates the expression of EGFRvIII in homogenized cell lysates from DU145vIII and DU145 prostate carcinoma cells. EGFRvIII is shown in cell lysates of DU145vIII cells which is lacking in parental DU145 cell lysates (Fig. 1A). DU145vIII cells may also incorporate the oncogenic EGFRvIII in the cargo of their membrane shed microvesicles (Fig. 1B). After collecting microvesicles from cell cultures in vitro, DU145vIII derived show the incorporation of EGFRvIII in the microvesicles fraction (Fig. 1B). Both DU145vIII and DU145 derived microvesicles show the presence of flotillin-1, a protein expressed within the membranes of microvesicles and commonly used as a marker for the microvesicle fraction. As the expression of EGFRvIII was previously illustrated in DU145vIII cells (Read et al., 2017), this analysis was additionally performed to show that cell lysates from DU145vIII still express EGFRvIII and that it can be detected in the derived microvesicles.
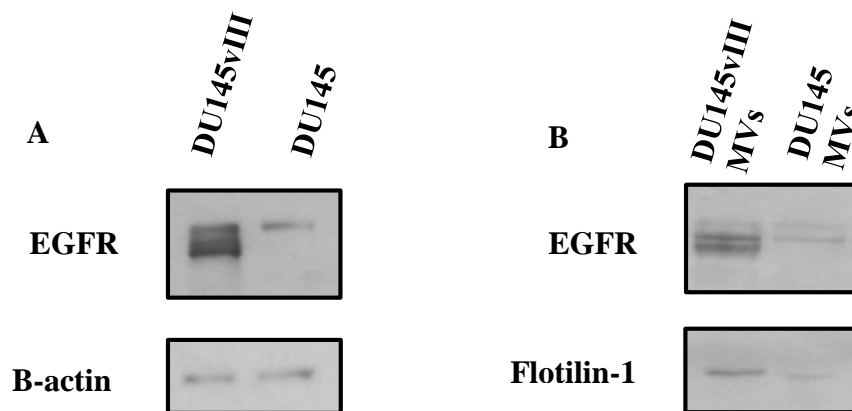
Figure 1. Expression of the oncogenic EGFRvIII in DU145vIII prostate carcinoma cells and microvesicles. (A) Cell lysates from DU145vIII cells show expression of EGFRvIII which is not present in DU145 cell lysates. (B) Microvesicles collected from DU145vIII also show the expression of EGFRvIII not present in microvesicles from DU145 cells.

3.2. Thrombin generation by microvesicles derived from prostate cancer cells is correlated with the oncogenic characteristics of cells.

To determine the procoagulant nature of prostatic cancer microvesicles, we used the chromogenic substrate Chromozym TH in order to assess the thrombin generation of microvesicles. Microvesicles from two prostate cancer cell lines were compared, those from the prostate carcinoma DU145 cell line and its DU145vIII variant containing the EGFRvIII oncogenic receptor (Al-Nedawi et al., 2008). This was done in order to investigate the effect of oncogenes to the procoagulant properties of their microvesicles. First, microvesicles derived from DU145vIII exhibited more procoagulant activity than DU145 derived MVs due to the increased ability of generating active Factor IIa (thrombin), which acts on the Chromozym TH substrate and increasing absorbance (Fig. 2).
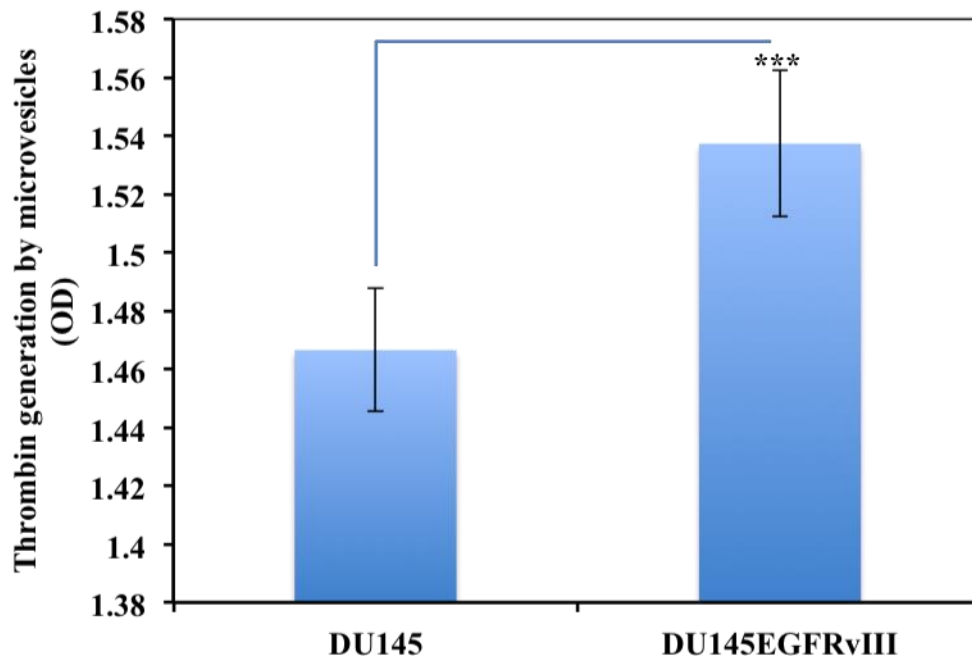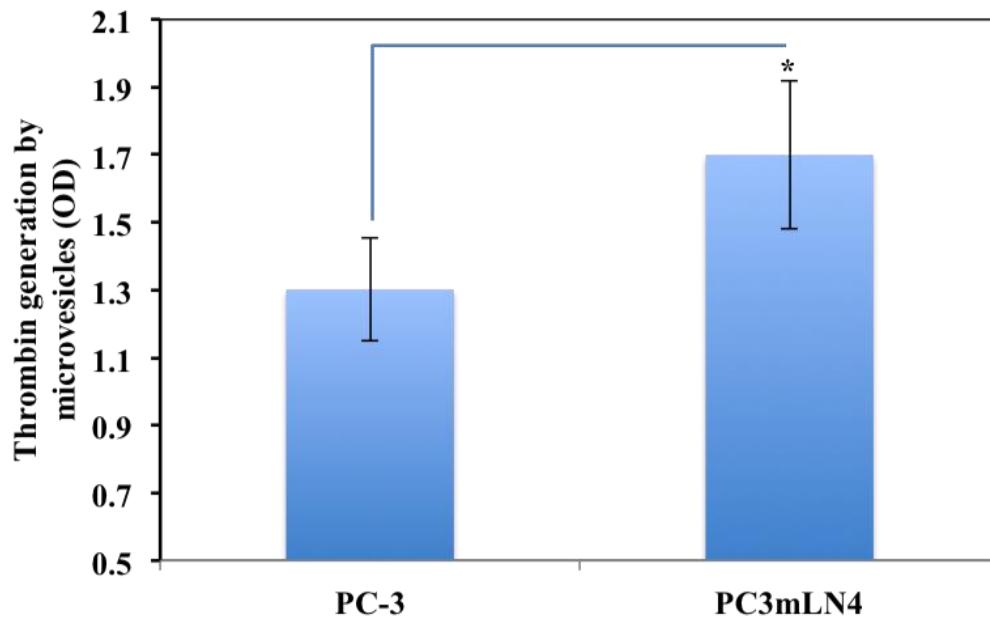
Figure 2. The effect of oncogenic characteristics on thrombin generation by

microvesicles extracted from prostate carcinoma cell lines. Microvesicles from

DU145vIII cells containing the oncogenic EGFRvIII showed a significant increase in

thrombin generation compared to microvesicles from the parental DU145 cells.

Microvesicles were incubated with 0.3 mM Chromozym TH substrate, 100 mM CaCl2

and 50 mM Tris-buffer. The increase in absorbance was measured at room temperature in

a continuous kinetic reading of 405 nm, according to the manufacturer's guidelines. Data

were optimised according to equivalent overall protein concentration of microvesicles

using a Bradford Protein Assay. The results represent the mean of three experiments $\pm$

SE. Differences from DU145 were shown to be significant (***: $p < 0.001$).

3.3. Thrombin generation by microvesicles derived from prostate cancer cells is correlated with the metastatic characteristics of cells.

We compared the procoagulant activity from epithelial prostate adenocarcinoma cell line PC3 and its more metastatic variant PC3-MLN4 (Glinsky et al., 2004). The PC3-MLN4 derived microvesicles showed a marked increase in the ability to generate active thrombin compared to PC3 MVs (Fig. 3). Together, these data suggest that the procoagulant activity (thrombin generation) of prostatic cancer microvesicles differ according to their metastatic potential.

Figure 3. The effect of metastatic characteristics of prostate cancer cell derived microvesicles on the thrombin generation. Microvesicles derived from the more metastatic prostate adenocarcinoma PC3-MLN4 had increased thrombin generation compared to microvesicles derived from PC3 cells. Microvesicles were incubated with 0.3 mM Chromozym TH substrate, 100 mM CaCl2 and 50 mM Tris-buffer. The increase in absorbance was measured at room temperature in a continuous kinetic reading of 405 nm, according to the manufacturer's guidelines. Data were optimised according to equivalent overall protein concentration of microvesicles using a Bradford Protein Assay. The results represent the mean of three experiments ± SE. Differences from DU145 were shown to be significant (*: $p < 0.05$).

3.4. Thrombin generation by microvesicles of prostate cancer patients' plasma is correlated with the disease progression.

We next wanted to investigate the direct procoagulant activity of microvesicles from prostate cancer patients, in order to investigate the direct relationship of microvesicles from different prostate cancer stages. Patient plasma samples were collected and microvesicles extracted. Tumour stages in patients differed in their extent of metastasis. In the organ confined (OC) stage, as the name suggests, refers to a tumour which is localized within the prostate organ with no metastasis. Extracapsular extension (EC) refers to the invasion of the external capsule of the prostate organ, and at this point is considered on its way for metastasis to other organs. Finally, the third stage, the seminal invasion state (SI), refers to a tumour that has spread to nearby seminal vesicles. Therefore, in a similar setup to microvesicles with cell cultures, we investigated the procoagulant activity of prostate cancer patients' plasma microvesicles after incubation with the Chromozym TH substrate. As seen in Figure 4, the procoagulant activity of MVs increases with the stage of prostate cancer in patients, correlating with the tumour's metastatic potential. These results further support the earlier results with cell culture microvesicles, that prostate cancer patient plasma microvesicles show an elevation of hypercoagulability based on the oncogenic and metastatic potential of the prostate tumour.
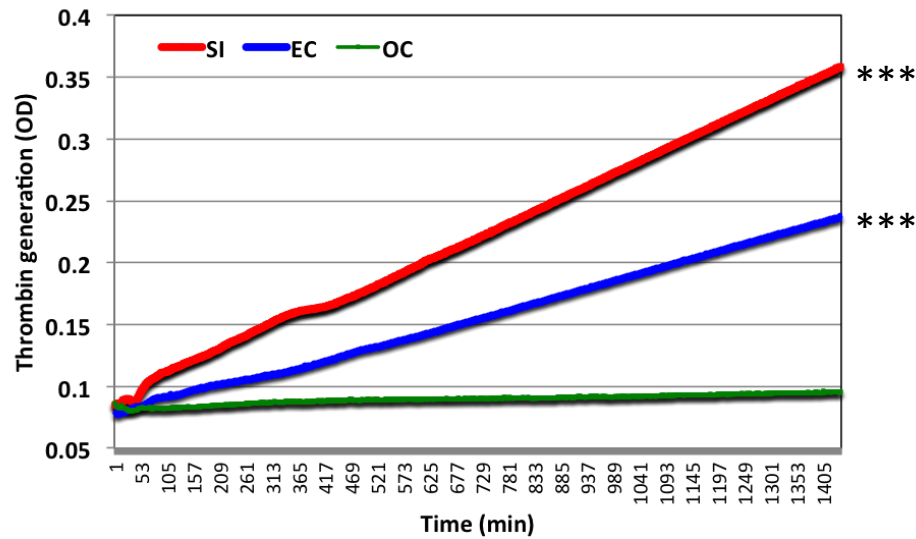
Figure 4. Thrombin generation in microvesicles extracted from prostate cancer patients'

plasma with different stages of tumour metastasis. OC: organ confined prostate tumour.

EC: extracapsular extension of the tumour, where it has invaded the layer of connective

tissue surrounding the prostate, the prostatic capsule. SI: seminal invasion, the prostate

tumour has spread to the seminal vesicles. Microvesicles extracted from patients at the SI

stage show more thrombin generation than EC-tumour-bearing patients. Patients with EC

tumours show increased thrombin generation compared to OC tumour in prostate cancer

patients. Microvesicle samples were incubated with 0.3 mM Chromozym TH substrate

with 100 mM CaCl2 and 50 mM Tris-buffer. The increase in absorbance was measured at

a continuous kinetic reading of 405 nm at room temperature. Data were optimised

according to equivalent overall protein concentration of microvesicles using a Bradford

Protein Assay. The results represent the mean of three experiments ± SE. Differences in

thrombin generation from the OC microvesicles were shown to be significant (***: $p <$

0.001).

3.5. Expression of the tagged-hPAR1 in CHO cells.

We have transfected the Chinese Hamster Ovarian epithelial cells with a plasmid of the human construct of the protease-activated receptor 1 (hPAR1). This hPAR1 construct contains a mCherry and an eYFP fluorescent protein tags on the N-terminus and C-terminus, respectively (Fig. 5). In order to assess the success of the tagged-hPAR1 transfection, the ATAP2 anti-PAR1 mouse monoclonal antibody was used to detect the tagged-hPAR1 in cell lysates of CHO (hPAR1). The ATAP2 antibody recognizes the SFLLRN sequence, which is the ligand motif imbedded within the receptor (Coughlin S.R., 2000). As can be seen in Figure 6A, the tagged hPAR1 is detected in the transfected CHO (hPAR1) cells but not in the CHO cell line, illustrating the successful transfection of the tagged hPAR1. In addition, tagged-hPAR1 can also be detected with the expression of anti-GFP antibody indicative of the eYFP fluorescent protein present on the C-terminus of the tagged-hPAR1 (Fig. 6A).
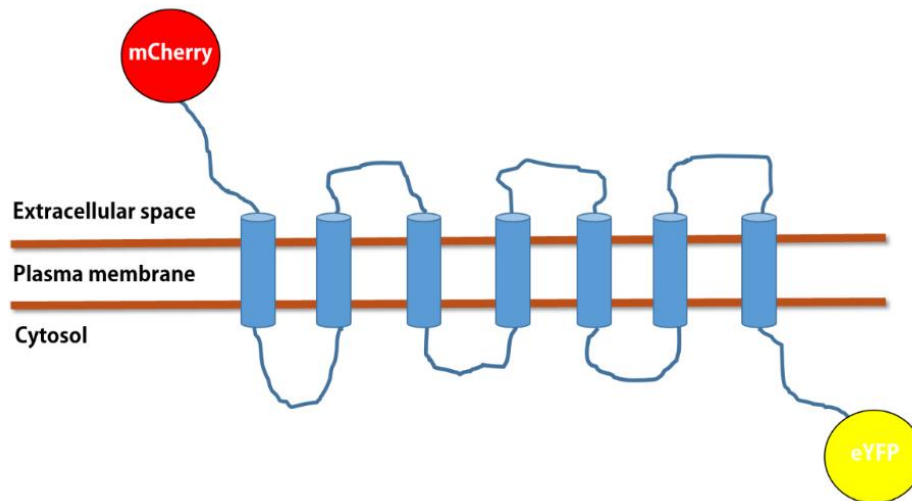
Figure 5. A diagram illustrating the location of the mCherry and eYFP fluorescent tags, located on the N-terminus and C-terminus of the dually tagged-hPAR1. The N-terminus, which acts as the main site of thrombin cleavage is located on the extracellular space along with the mCherry fluorescent protein.

A

PAR1

Anti-GFP

B-actin

B

P-ERK 1/2

ERK 1/2

Phospho-p38
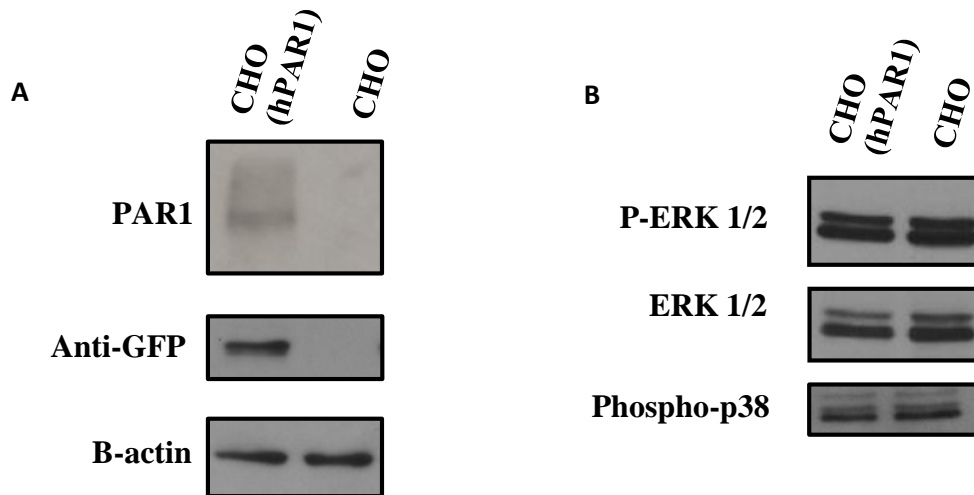
Figure 6. Transfection of the CHO cell line with tagged-hPAR1. (A) An immunoblot showing the expression of the tagged-hPAR1 in transfected CHO (hPAR1) with the anti-PAR1 antibody (ATAP2), which is not present in non-transfected CHO cells, indicating the successful transfection with the tagged-hPAR1. Anti-GFP indicates the expression of tagged-hPAR1 through the detection of the eYFP fluorescent protein attached on the C-terminus of tagged-hPAR1. (B) Impact of the tagged-PAR1 receptor on the stimulation of ERK 1/2, showing no effect of stimulation due to the presence of the tagged-hPAR1 receptor alone.
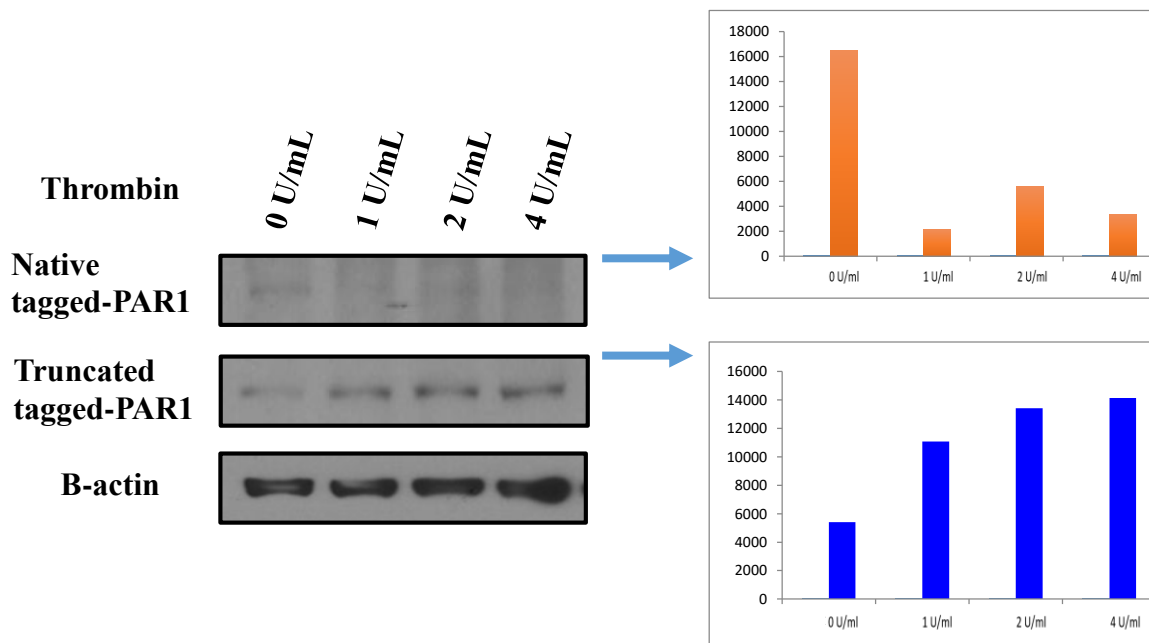
3.6. Activation of tagged-hPAR1 by thrombin.

To assess the activation of the tagged hPAR1 in CHO (hPAR1) cells, thrombin, the primary activator of hPAR1, was used. Thrombin is a serine protease enzyme that acts on Arginine residues Arg41 and Arg42 of PAR1, leading to the truncation and removal of the N-terminus in PAR1, and the subsequent downstream signalling (Coughlin S. R., 2000). We examined the truncation and activation of tagged hPAR1 by thrombin through the protein expression of both native and truncated forms of tagged-hPAR1. Cultured CHO (hPAR1) were incubated with thrombin at increasing concentrations, then their lysates were immunoblotted using anti-PAR1 monoclonal antibody (ATAP2). Figure 7 shows the effect of thrombin on the loss of detected native tagged-hPAR1, which is almost diminished following the first treatment with thrombin. This is explained by thrombin's action on tagged-hPAR1, which cleaves the N-terminus leading to a reduction in the size of the native form of the receptor, and thus the subsequent loss of detection of tagged-hPAR1 in the treatment lanes as compared to the control lane.

Furthermore, after cleavage of the native form of tagged-hPAR1, the ATAP2 anti-PAR1 antibody also detects the truncated version of tagged-hPAR1. Due to the truncated version of tagged-hPAR1 being smaller in size compared to the native form, it is detected at a lower molecular weight range. Figure 7 illustrates the corresponding increase in truncated tagged-hPAR1 expression as the native form detection is decreased with thrombin treatment. Both the reduction in the native form and increase in truncated form indicate the cleavage of the extracellular N-terminus, providing evidence of activation of tagged-hPAR1 by thrombin.

Next, we further examined thrombin's activation of tagged-hPAR1 using spectrophotometry to detect the mCherry fluorescent protein tag. The transfected CHO (hPAR1) cells were grown in a cell culture well plate, treated with thrombin then read for mCherry in a spectrophotometer for both the surrounding supernatant and the cultured cells. The surrounding medium of cultured cells was collected after incubation with thrombin and measured for the mCherry fluorescence. As thrombin concentrations increased, the detection of mCherry fluorescence increased gradually (Fig. 8A). This observation suggests the active truncation and release of the N-terminus along with the mCherry fluorescence into the supernatant. On the other hand, the mCherry fluorescence decreased significantly when read in cultured cells on the first treatment with thrombin (1U /mL), but with a lesser extent upon subsequent treatments (Fig. 8B). This indicates thrombin's activity on cleaving and releasing the N-terminus exodomain containing mCherry away from the tagged-hPAR1 receptor.

Thus, this data illustrates the impact of the thrombin enzyme on the cleavage and activation of the tagged-hPAR1 located on the cellular surface of cells. Overall, these data demonstrate the expression of functional tagged hPAR1 in the CHO (hPAR1) cells, and that it has an active role as a thrombin receptor.

Figure 7. Assessment of tagged-PAR1 activation with thrombin using Western Blots for detection of native and truncated tagged-hPAR1. (A) Using the ATAP2 antibody against PAR1, the detection of native tagged-hPAR1 is significantly reduced upon increasing treatment with thrombin. As thrombin acts on the N-terminus leading to its removal from the receptor, tagged-PAR1 becomes smaller in size and its detection is therefore reduced when compared to the full size tagged-hPAR1 in the control lane. (B) The same ATAP2 anti-PAR1 antibody was used to detect the truncated version of tagged-hPAR1 at a reduced molecular weight region. The reduced detection of the native form of tagged-hPAR1 is accompanied by the increase in the expression of the truncated form of tagged-hPAR1. Histograms are obtained using ImageJ software and represent the optical densities of bands.

A

B
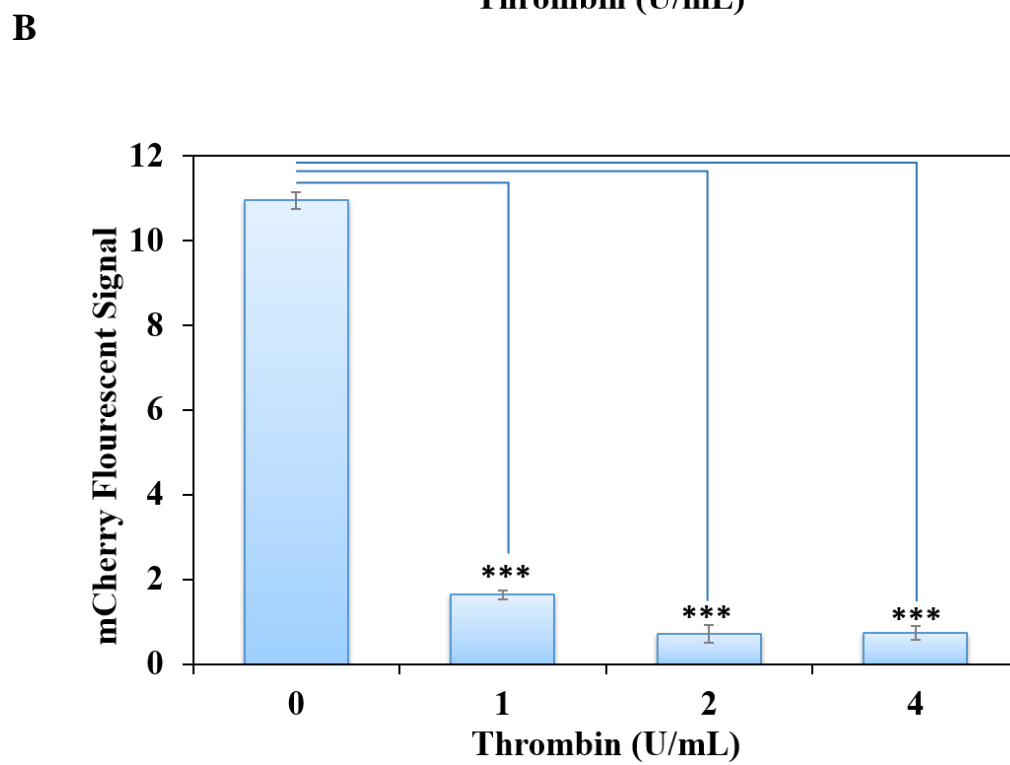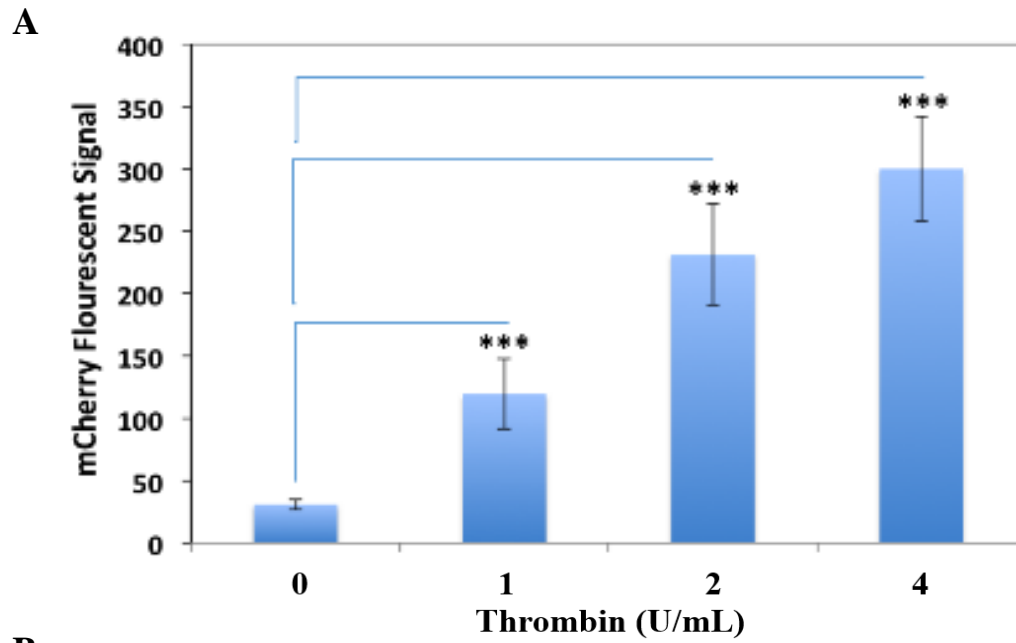
Figure 8. Assessment of tagged-hPAR1 activation with thrombin by utilizing the

mCherry fluorescent tag located on the N-terminus of tagged-PAR1. (A) The supernatant

surrounding cultured cells was collected and read for the mCherry fluorescence.

Treatment with thrombin shows the increase in detected mCherry, suggesting thrombin's

action on tagged-hPAR1 by cleaving the N-terminus containing mCherry into the

collected supernatant. (B) Cultured CHO (hPAR1) cells were read for the mCherry

signal, and show a significant drop in the detected signal following the first treatment

with thrombin (1 U/mL). This indicates the cleavage and removal of the tagged-hPAR1

N-terminus containing mCherry from the cells. The mCherry signal was detected using a

fluorescent spectrophotometer at excitation and emission wavelengths of 587 nm and 610

nm, respectively. The results represent the mean of three experiments $\pm$ SE. Differences

from the control (0 U/mL of thrombin) are shown as significant (***: $p < 0.001$).

3.7. Activation of hPAR1 signalling pathway by thrombin.

To further examine the effects of thrombin on the tagged hPAR1 in transfected CHO (hPAR1) cells, we assessed the expression of the extracellular signal regulated protein kinases (ERK) in CHO (hPAR1) cells treated with thrombin. Figure 9A illustrates the effect of thrombin on P-ERK 1/2, which resulted in a distinctive pattern of deactivation as thrombin concentrations were increased (Figure 9A). Furthermore, to investigate the dependency of thrombin's downstream signalling deactivation to the tagged-hPAR1 transmembrane protein, non-transfected CHO cells were treated with thrombin using the same setup as with thrombin treatment. Figure 8B shows the effect on P-ERK stimulation and expression of ERK in thrombin treated non-transfected CHO cells. Thrombin resulted in no impact on P-ERK at any concentration of thrombin (Fig. 9B). This illustrates that the deactivation of P-ERK was dependent on the interaction between thrombin and the tagged-hPAR1.

A similar trend is observed in phospho-AKT, where P-AKT activation is decreased significantly at 1 U/mL of thrombin and at subsequent treatment concentrations (Fig. 10). In addition, phospho-p38 is decreased on treatment with increasing concentrations of Thrombin (Fig. 10). Since P-ERK and phospho-p38 activation are involved to promoting growth of cells (Johnson et al., 2002) their deactivation by thrombin suggests its role in suppressing cellular proliferation and growth through its actions on tagged-PAR1. On the other hand, P-AKT is responsible for inhibiting apoptosis in cancer cells promoting cell survival (Brunet et al., 1999), thus thrombin

produces a combined effect of suppressing cellular growth and reducing cell survival by

deactivating P-ERK and P-AKT protein kinases.
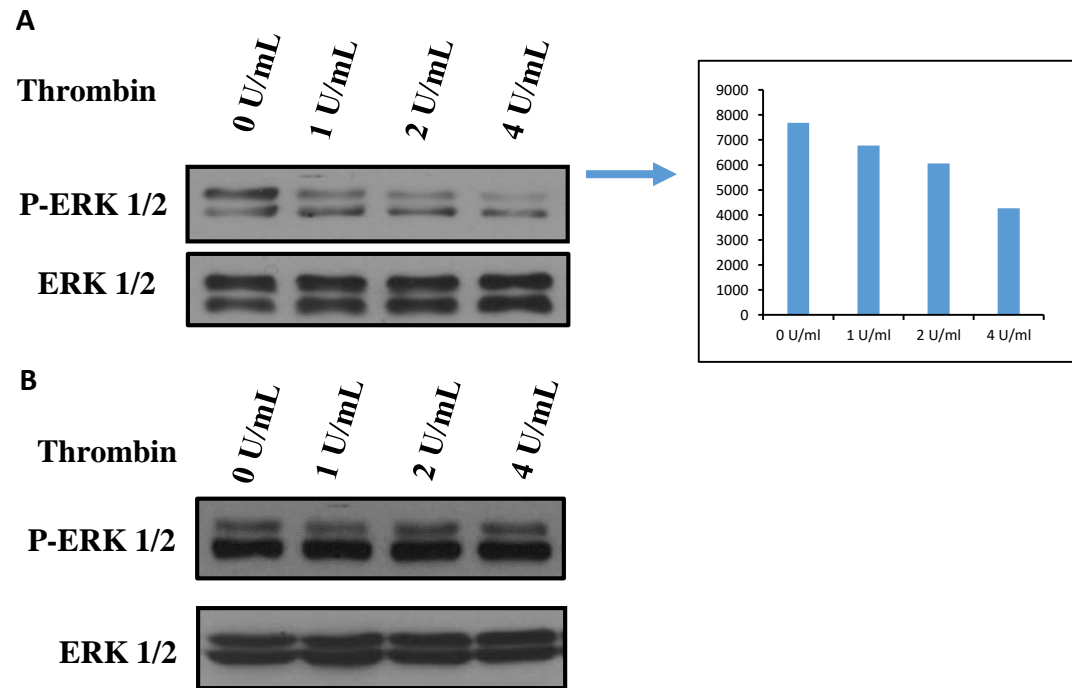
Figure 9. Effect of thrombin on the cellular proliferation pathway as a result of tagged-PAR1 activation by thrombin. (A) Transfected CHO (hPAR1) cells were incubated with increasing doses of thrombin. Cells were lysed and immunoblotted for antibodies against P-ERK 1/2 and total ERK 1/2. Stimulation of P-ERK 1/2 is gradually decreased upon increasing doses with thrombin. (B) Non-transfected CHO cells were treated with thrombin in the same manner as with CHO (hPAR1) cells, and their cell lysates were immunoblotted for P-ERK 1/2 and total ERK 1/2. There was no change in P-ERK 1/2 activation in non-transfected CHO cells. This indicates the dependency of P-ERK 1/2 deactivation on the truncation of tagged-PAR1 by thrombin. Histograms are obtained using ImageJ software and represent the optical densities of the bands.
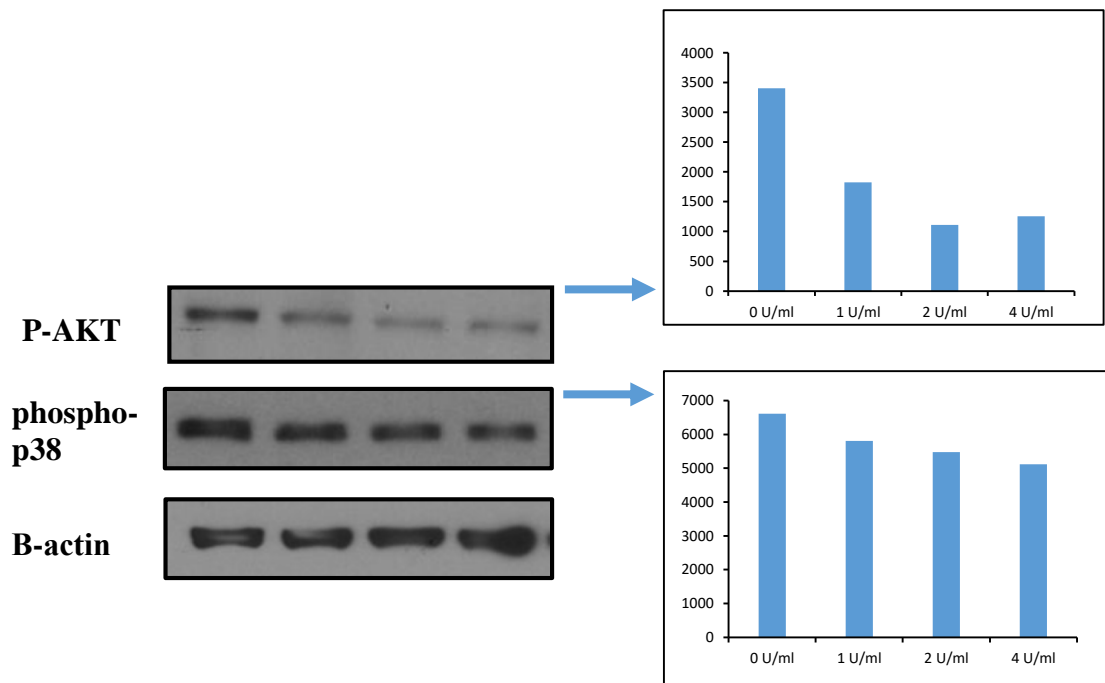
Figure 10. Effect of thrombin on the cell survival pathway as a result of tagged-PAR1 activation by thrombin. Transfected CHO (hPAR1) cells were incubated with thrombin at increasing doses. Cells were lysed and immunoblotted for antibodies against P-Akt and phospho-p38. Phospho-Akt activation is decreased in a dose-dependent manner with thrombin. A similar trend is observed in decreased phospho-p38 activation. Histograms are obtained using ImageJ software and represent the optical densities of the bands.

3.8. Truncation of hPAR1 by microvesicles derived from DU145-EGFRvIII.

We next investigated the procoagulant activity of prostate cancer microvesicles in a similar experimental setup to that of thrombin, and this was done in order to investigate their thrombin generation and interactions with tagged-hPAR1. Transfected CHO (hPAR1) cells were incubated with DU145vIII derived microvesicles at various concentrations of 0 µg/mL, 100 µg/mL and 200 µg/mL. Figure 11 shows the effect of DU145vIII microvesicles on the loss of detection of native tagged-hPAR1, which is almost diminished upon the first treatment concentration. This effect illustrates thrombin's action of cleaving the tagged-hPAR1's N-terminus, which reduces the size of the receptor, causing its detection to be diminished when compared to the full-size form found in the control lane. This data further suggests that prostate carcinoma DU145vIII derived microvesicles generate active Factor IIa (thrombin).

Additionally, the thrombin generation by DU145vIII-derived microvesicles was further investigated through their action on tagged-hPAR1 by using confocal microscopy, as a detection method of the mCherry and eYFP fluorescent tags. CHO (hPAR1) cells were grown in a chamber slide then treated with microvesicles at 200 µg/mL. Following treatment, cells were fixed and observed under a confocal microscope for the measurement of the mCherry and eYFP fluorescent signals. Figure 12 illustrates the effect of incubation with DU145vIII microvesicles on the detection of eYFP and mCherry signals in CHO (hPAR1) cells. The top panel indicates equivalent fluorescent signals from the eYFP and mCherry flourophores, which is shown in the merge column as a yellow colour indicating overlap of both fluorescent signals. On the bottom panel,

the detection of mCherry is relatively decreased when compared to the eYFP

fluorescence. This suggests the active truncation and release of the N-terminus containing

mCherry as thrombin is generated from the DU145vIII derived microvesicles (Fig. 12).

Furthermore, spectrophotometric analysis was performed on the transfected CHO

(hPAR1) cells incubated with DU145vIII microvesicles in order to further elucidate the

procoagulant thrombin activity of MVs on the dually tagged-hPAR1. Similarly to the

thrombin treated CHO (hPAR1), incubation with DU145vIII derived MVs resulted in the

increase of mCherry fluorescent signal in the supernatant surrounding cells. This increase

of detected mCherry is explained in the cleavage and removal of the mCherry tagged N-

terminus exodomain into the surrounding media of treated cells (Fig 13). The results

mimic the effects observed with thrombin treatment, providing a quantitative method

which further supporting that DU145vIII derived microvesicles generate active thrombin

which acts on the tagged-hPAR1 located on the surface of CHO (hPAR1) cells.
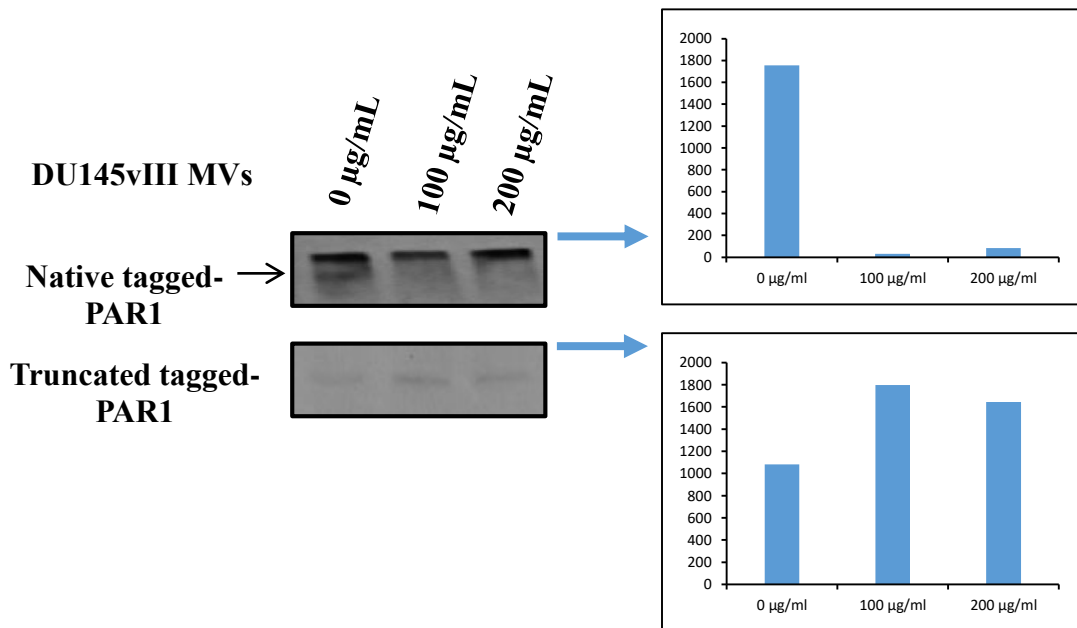
Figure 11. The effect of treating CHO (hPAR1) cells with DU145vIII-derived microvesicles on the expression of tagged-hPAR1. Western blot analysis of CHO (hPAR1) lysates treated with DU145vIII MVs, immunoblotted for the ATAP2 anti-PAR1 antibody, shows the loss of signal of native tagged-hPAR1 with increasing treatment concentrations. The loss of signal in the native form of tagged-hPAR1 is accompanied by the gain of signal in the truncated region of tagged-hPAR1, similarly as observed in the thrombin treated CHO (hPAR1) cells. Histograms are obtained using ImageJ software and represent the optical densities of bands.
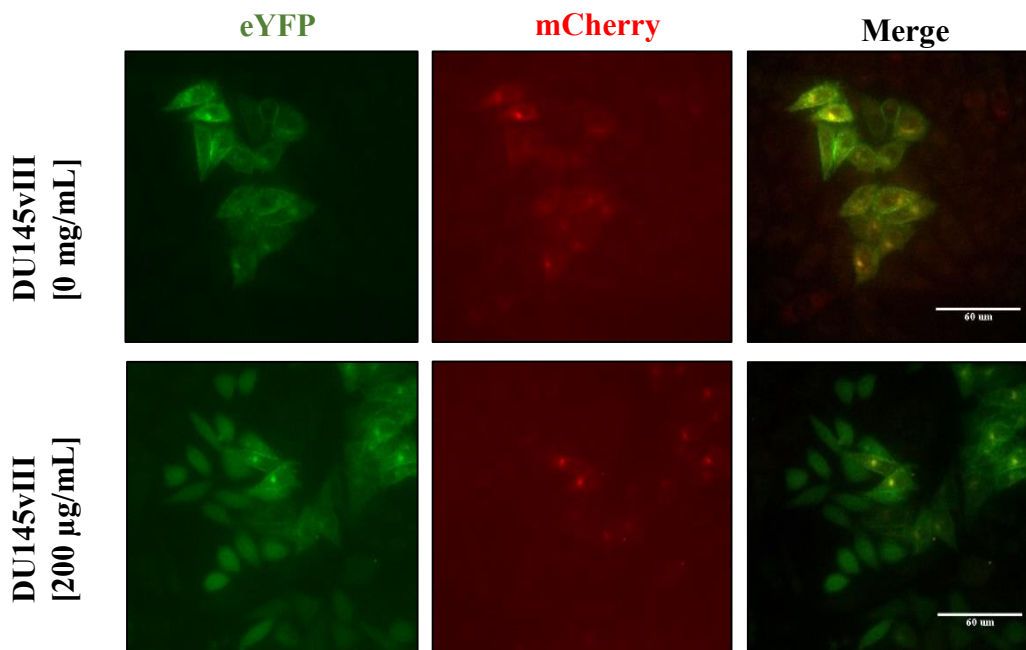
Figure 12. Activation of CHO (hPAR1) by thrombin generated by DU145vIII-derived

microvesicles. Top panel: no incubation with DU145vIII derived microvesicles. Both the

mCherry and eYFP signals as detected by TexasRed and FITC respectively show

equivalent flourescent signal. Bottom panel: incubation with 200 µg/mL of DU145vIII-

derived microvesicles. The TexasRed signal is reduced compared to FITC, indicating the

loss of the mCherry fluorescence on the N-terminus as a result of thrombin activation on

tagged-hPAR1.
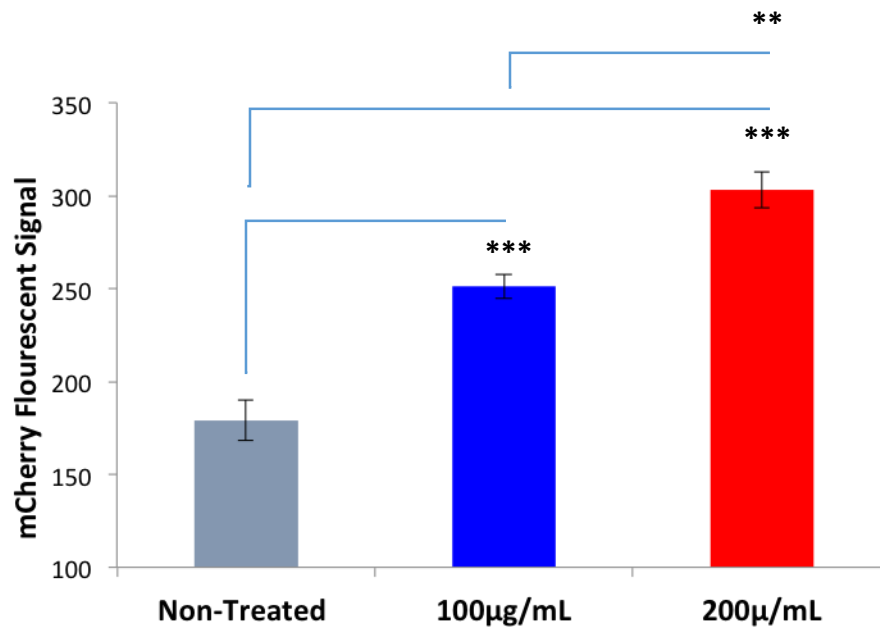
Figure 13. Effect of DU145vIII-derived microvesicles on the change of mCherry

detected signal as a result of tagged-PAR1 activation. Transfected CHO (hPAR1) cells

were incubated with microvesicles derived from DU145vIII cell cultures at two

concentrations of 100 µg/mL and 200 µg/mL. The supernatant of treated cells was

collected and measured for the mCherry fluorescent signal. Increased concentrations of

DU145vIII-derived MVs resulted in the gradual increase of detected mCherry signal. The

mCherry signal was detected using a fluorescent spectrophotometer at excitation and

emission wavelengths of 587 nm and 610 nm, respectively. The results represent the

mean of three experiments ± SE. Differences from the non-treated and in between

treatments were shown to be significant (***: $p < 0.001$ and **: $p < 0.01$, respectively).

CHAPTER FOUR: DISCUSSION

Cancer patients present with a significantly increased risk of developing VTE (Walker et al., 2013). The term Trousseau's syndrome was used to refer to any haemostatic abnormality caused by cancer (Varki et al., 2007). Clinically, Trousseau's syndrome is most commonly manifested through migratory venous thromboembolism, which is associated with increased mortality and healthcare costs among cancer patients (Shirvanian and Tapson, 2015). The incidence of migratory venous thromboembolism is increased in cancer patients, and such thrombotic events may precede the diagnosis of malignancy (Varki et al., 2007). Although there is considerable research on the role of coagulation in cancer, little is understood with regards to the mechanism of thrombus formation at distant sites from a tumour. However, cancer cell-derived extracellular vesicles have been studied in relation to the link between cancer and migratory thrombosis. Tumour-derived vesicles have been implicated in many cancerous properties, including promoting growth, angiogenesis, metastasis and evasion of the immune system (Al-Nedawi et al., 2008, Skog et al., 2008, Valenti et al., 2006 & Graves et al., 2004).

In order to provide a possible mechanism for migratory venous thromboembolism in prostate cancer patients, we investigated the pro-coagulant activity of tumour-derived microvesicles from prostate cancer cell lines. Compared to DU145 cell-derived microvesicles, microvesicles from the more aggressive DU145vIII cells exhibited greater thrombin generation (Fig. 2). In addition, microvesicles from the highly metastatic PC3-MLN4 cells had greater thrombin generation than PC-3 cell-derived microvesicles (Fig. 3). Thrombin is the principal product of the coagulation pathway, and has been

implicated in many vascular endothelial responses, including inflammatory, endothelial cellular activation and angiogenesis (Nierodzik and Karpatkin, 2006). We demonstrated the ability of prostate cancer-derived vesicles to generate thrombin, and this ability was related to the increased metastatic properties of prostate cancer cells. In addition, the constitutively active oncogenic receptor EGFRvIII, promotes various cancerous responses such as cellular proliferation and differentiation (Al-Nedawi et al., 2008). We show that in MVs obtained from the DU145vIII prostate carcinoma cells, the increased thrombin generation was linked to the oncogenic EGFRvIII (Fig. 2). This suggests a role of oncogenes in regulating the procoagulant activity of tumour-derived microvesicles, indicating a link between cancer progression and Trousseau's syndrome.

Considerable research reports on tumour-derived microvesicles containing TF. Tissue factor is a cell surface glycoprotein, which initiates the extrinsic pathway of the coagulation cascade, and its overexpression within tumours is correlated with advanced grades of cancer and cancer development (Kakkar et al., 1995 & Meuller et al., 1992). Cancer cells release TF-positive microparticles that exhibit strong pro-coagulant activity, and are associated with an increased incidence of VTE as well as increased mortality among cancer patients (Hernandez et al., 2013). Vessel injury in mouse models demonstrate an accelerated rate of thrombus formation when TF+ microvesicles are injected into the circulation (Thomas et al., 2015). These studies illustrate the strong association between the incidence of thrombus formation and the presence of TF+ microvesicles in the circulation.

As we demonstrated the active role of thrombin generation by prostate cancer-cell derived microvesicles, we further investigated the impact of microvesicles on the dually tagged-hPAR1 as a useful measure of thrombin's activation on PAR1. Due to the presence of the mCherry and eYFP fluorescence proteins on the N-terminus and C-terminus, respectively, we can determine the cleavage of the N-terminus through changes in the mCherry fluorescence. Both thrombin and prostate cancer-derived MVs demonstrated an increase of the mCherry signal in the supernatant of treated CHO (hPAR1) cells, while causing a concomitant decrease of signal on the adherent cells (Figs. 8B and 13). These findings suggest thrombin's action of truncating the N-terminus and releasing its associated mCherry fluorescence. Both the thrombin generation assay and the utilization of fluorescent signals indicate the independent pro-coagulant activity of microvesicles to generate functional thrombin enzyme, which acts on PAR1 leading to activation of downstream signalling pathways.

In addition to cell culture derived microvesicles from prostate cancer cells, we also assessed the pro-coagulant activities of microvesicles extracted from the plasma of prostate cancer patients. Thrombin generation was greatly increased in tumour-derived microvesicles of those patients with a metastasized prostate tumour, compared to a more benign tumour (Fig. 4). Our study indicates a correlation between the extent of metastasis and the pro-coagulant activity of prostate cancer-derived microvesicles. This might suggest that the hyper-coagulation state in prostate cancer patients is essential or a reflection of cancer progression. For instance, as we demonstrated with prostate cancer cell lines, the presence of the oncogenic EGFRvIII in DU145vIII cell-derived MVs

promoted the ability to generate functional thrombin (Fig. 2). In addition, it was

previously shown that the introduction of EGFRvIII in the epidermal carcinoma A431

cells led to the induction of the vascular epidermal growth factor (VEGF) and promoted

angiogenesis (Al-Nedawi et al., 2009). Furthermore, the loss of PTEN tumour suppressor

also led to the up-regulation of TF expression in glioma cells exposed to hypoxic

conditions (Rong et al., 2005). The epithelial to mesenchymal transition in the epidermal

carcinoma A431 cells for instance, was enhanced with TF expression from activated

EGFR (Milsom et al., 2008). A clear trend emerges suggesting that the coagulation

cascade contributes to the progression of angiogenesis and the malignant phenotype in

cancer. On the other hand, cancerous pathways may regulate the expression of TF

through angiogenesis. Metastatic breast tumours express more TF in the vascular

endothelial cells embedded within the tumour and is correlated with the degree of

metastasis (Contrino et al., 1996). Therefore, the extent of cancer cellular proliferation is

reflected with amplified pro-coagulant activities in tumour cells. In this regard, more

extensive studies are required for identifying the players involved in the mechanism

between the activation of coagulation and promoting malignancy.

Furthermore, in order to understand how thrombin generating microvesicles exert

their various effects of promoting invasiveness, metastasis and growth, we investigated

their pro-coagulant role with regards to PAR1 activation. First, purified thrombin was

able to deactivate phospho-ERK 1/2 in transfected CHO (hPAR1) cells in a dose-

dependent manner (Fig. 9). Additionally, the same pattern of dose-dependent deactivation

was observed with P-Akt and phospho-p38 (Fig. 10). In light with these findings, studies

report that the activation of PAR1 in tumour cells promotes tumour invasion, cell migration and angiogenesis (Tsopanoglou et al., 1993; Darmoul et al, 2003). Contrary to such studies in cancer cells in which the MAPK pathway is activated with thrombin, our results indicate a reversed role of thrombin mediated PAR1 deactivation, and possibly reduced tumour functions due to inhibition of MAPK. However, our results of P-ERK, P-Akt and phospho-p38 deactivation were possibly dependent on the activation of tagged-hPAR1 alone, while outside of the context of cancer cells. Therefore, our results might suggest that PAR1 in cancer cells is activated in co-ordination with other receptors/factors that result in the stimulation of P-ERK. This merits further investigation into the exact role of thrombin-mediated PAR1 activation in cancer cells and its effect on tumour progression.

Taken together, our findings suggest that prostate cancer-cell derived microvesicles can independently generate the active serine protease enzyme thrombin, suggesting their capacity to contain the molecules of the coagulation cascade required for thrombin formation. In addition, active thrombin generated by prostate cancer-cell derived microvesicles illustrates a potential role for microvesicles to regulate cancerous properties through tagged-hPAR1 activation.

CHAPTER FIVE: CONCLUSIONS AND FUTURE DIRECTIONS

Our results illustrate the pro-coagulant properties of prostate cancer-cell derived microvesicles, with the ability of independently generating active thrombin. We have shown that thrombin generation correlates with the degree of oncogenic potential and metastasis of prostate cancer cells. Microvesicles from the prostate carcinoma cell line harbouring the oncogenic EGFRvIII, DU145vIII, as well as from the highly metastatic prostate adenocarcinoma PC3-MLN4 cells demonstrated increased thrombin generation compared to MVs from the DU145 and PC3 cell lines (Fig. 2 and 3). Similarly, the thrombin generation from PC patient plasma microvesicles was associated with the degree of metastasis of their corresponding prostate tumours. These findings suggest a role of prostate tumour-derived microvesicles in the cancer-associated thrombosis observed in prostate cancer patients.

Another significant finding in this study was the role of pro-coagulant prostate cancer-derived microvesicles in activating tagged-hPAR1. Thrombin generated by prostate cancer-cell derived microvesicles can directly cleave tagged-hPAR1. As PAR1 is expressed in tumour cells and the vascular endothelium, our findings further suggest a function of prostate cancer MVs in promoting the cancerous phenotype, by activating PAR1 and possibly regulating various tumour functions.

Also, our observation that thrombin's action on PAR1 in CHO (hPAR1) cells and outside the context of cancer cells, lead to the reduced activation of the MAPK signalling pathway, rather suggests that other factors are involved in the propagation of thrombin's

signal in cancer. This combined effect of multiple factors could be investigated through the use of inhibitors. For instance, inhibiting thrombin when incubating cells with prostate cancer-derived MVs could shed light on specific mechanisms of the role of PAR1 independently of other receptors. Ultimately, investigating the relationship of prostate cancer-cell derived microvesicles in the activation of the endothelium would provide insight into their potential of contributing to prostate tumour angiogenesis.

Upon understanding the role of tumour-derived microvesicles in the development of Trousseau's syndrome, it is important to investigate the potential therapeutic strategies against cancer-associated thrombosis. If tumour-derived microvesicles can independently lead to the formation of active thrombin, presumably, the tumour-derived microvesicles can be targeted for clearance or inhibition in the circulation to reduce incidence of venous thromboembolism.

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