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Role of Thrombin-Activatable Fibrinolysis Inhibitor in Breast Cancer Metastasis and Angiogenesis

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Role of Thrombin-Activatable Fibrinolysis Inhibitor in Breast Cancer Metastasis and Angiogenesis

by

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Role of Thrombin-Activatable Fibrinolysis Inhibitor in Breast Cancer Metastasis and Angiogenesis

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DECLARATION OF CO-AUTHORSHIP AND PREVIOUS PUBLICATION

I hereby declare that this dissertation incorporates material that is result of joint research, as follows:

This dissertation incorporates the outcome of joint research in collaboration with Jennifer Balun, Danielle Lanoue, Mouhanned El-Youssef, Rocco Romagnuolo, Janice Tubman, Dr. Lisa Porter and Dr. Cavallo-Medved under the supervision of Dr. Michael Boffa. In all cases, key ideas, primary contributions, experimental design, execution, data analysis, and interpretation were performed by the author (with exceptions listed below). Additionally, manuscripts were written initially by the author and revised and edited by Dr. Michael Boffa, Dr. Lisa Porter and Dr. Cavallo-Medved for Chapter 2, Dr. Michael Boffa, Jennifer Balun, Dr. Lisa Porter and Dr. Cavallo-Medved for Chapter 3 and revised and edited by Dr. Michael Boffa, for Chapter 4.

- Collaboration with Jennifer Balun is covered in Chapters 3 and 4 of this dissertation. For Chapter 3, Jennifer Balun performed experiments and data analysis for the following: cell invasion, tube formation, proteolysis and zymography experiments with PTCI and VEGF. For Chapter 4, Jennifer performed experiments and data analysis for the following: tube formation and proteolysis experiments with endothelial cells and co-culture experiments with endothelial cells and breast cancer cells.
- Collaboration with Danielle Lanoue is covered in Chapter 2 and Chapter 4 of this dissertation. For Chapter 2, Danielle Lanoue performed invasion and migration experiments with TAFI-CIIYQ. For Chapter 4, Danielle Lanoue assisted with molecular cloning of TM EGF3-6 mutants.
- Collaboration with Mouhanned El-Youssef is covered in Chapter 2 of this dissertation; this was through contribution of data analysis for proteolysis assays.
- Collaboration with Rocco Romagnuolo is covered in Chapter 2 of this dissertation; this was through intellectual contribution and experimental design for plasminogen activation assays.

- Collaboration with Janice Tubman is covered in Chapter 2 of this dissertation; this was through sample preparation.
- Collaboration with Dr. Lisa Porter is covered in Chapters 2, 3 and 4 of this dissertation; this was through intellectual contribution and joint funding.
- Collaboration with Dr. Cavallo-Medved is covered in Chapters 2, 3 and 4 of this dissertation; this was through intellectual contribution and joint funding.

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This dissertation includes 3 original manuscripts that have been previously published, submitted, or are in preparation for publication in peer reviewed journals, as follows:

Dissertation Chapter	**Publication Title and Full Citation**	**Publication Status**
Chapter 2	Bazzi ZA, Lanoue D, El-Youssef M, Romagnuolo R, Tubman J, Cavallo-Medved D, Porter LA, Boffa MB. Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) attenuates breast cancer cell metastatic behaviors through inhibition of plasminogen activation and extracellular proteolysis. *BMC Cancer.* 2016 May 24;16(1):328.	Published
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Chapter 4	Bazzi ZA, Balun J, Lanoue D, Cavallo-Medved D, Porter LA Boffa MB. Thrombomodulin as a cofactor for TAFI and protein C activation: role in breast cancer metastatic behaviours and angiogenic potential.	In preparation

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ABSTRACT

Cancer metastasis is an important process in cancer progression. This process is facilitated by proteases that promote degradation of the extracellular matrix, cell migration and cell invasion. Angiogenesis is important to the metastatic process and also involves extracellular matrix degradation, endothelial cell migration and invasion. Proteases such as plasmin and matrix metalloproteinases are responsible for degradation of the extracellular matrix, facilitating cancer cell invasion and subsequent metastasis. These proteases are also important in promoting tumour angiogenesis. Therefore, developing methods to target these proteases may effectively inhibit cancer metastasis and tumour angiogenesis. TAFI is a plasma zymogen initially known for its role in attenuating fibrinolysis. TAFIa is formed through cleavage by thrombin, plasmin or, by thrombin in complex with thrombomodulin. TAFIa is a carboxypeptidase, cleaving carboxyl terminal lysine residues from plasminogen binding sites on cell surface receptors, which are important in accelerating plasminogen activation to plasmin. We have demonstrated that TAFIa is able to inhibit metastatic behaviours, including breast cancer cell invasion, migration and collagen degradation. We have shown that TAFIa inhibits these metastatic behaviours through attenuation of plasminogen activation to plasmin. Additionally, we have demonstrated that TAFIa also acts as an anti-angiogenic factor, inhibiting endothelial cell invasion, migration, tube formation and collagen degradation. We have also shown that these effects are mediated by the ability of TAFIa to inhibit plasminogen activation on the endothelial cell surface. Taken together these results indicate that TAFIa is an anti-metastatic and anti-angiogenic factor that may represent a novel therapeutic strategy to target cancer metastasis.

To the loved ones I lost during my time as a graduate student,

my grandmothers Fatima & Noor Al-Sabah

and my uncle Yasser.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AAFR	N-(4-methoxyphenylazoformyl)-Arg-OH · HCl
APC	activated protein C
apoE	apolipoprotein-E
APP	amyloid precursor protein
bFGF	basic fibroblast growth factor
BME	basement membrane extract
BSA	bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
CM	conditioned media
CPR	arginine carboxypeptidase
CPU	unstable carboxypeptidase
CTCs	circulating tumour cells
DQ	dye-quenched
EBM-2	endothelial cell basal medium-2
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
FBS	fetal bovine serum
FDPs	fibrin degradation products
FGFR1	type 1 fibroblast growth factor receptor
GEMSA	2-guanindinoethylmercaptosuccinic acid

GRE	glucocorticoid responsive element
HBS	HEPES-buffered saline
HMGB1	high mobility group box 1
HNF-1α	hepatocyte nuclear factor-1α
HRT	hormone replacement therapy
HUVECs	human umbilical vein endothelial cells
IL	interleukin
LPS	lipopolysaccharide
MERGEPTA	2-mercaptomethyl-3-guanindinoethylthiopropanoic acid
MMP	matrix metalloproteinase
NF-Y	nuclear factor-Y
PAI	plasminogen activator inhibitor
PAS	plasminogen activation system
PBS	phosphate buffered saline
PC	protein C
PEG	polyethylene glycol
PI3K	phosphatidylinositol-3-kinase
Plg	plasminogen
Plg-R	plasminogen receptor
Pln	plasmin
PPACK	D-phenylalanyl-prolyl-argininyl chloromethyl ketone
proCPB	procarboxypeptidase B
PTCI	potato tuber carboxypeptidase inhibitor

rTM	recombinant thrombomodulin
S-2366	pyroglutamyl-prolyl-argininyl-p-nitroanilide hydrochloride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SERMs	selective estrogen receptor modulators
SERPINS	serine protease inhibitor superfamily
SNP	single nucleotide polymorphism
TAFI	thrombin-activatable fibrinolysis inhibitor
TAFIa	activated thrombin-activatable fibrinolysis inhibitor
TAFIai	inactivated thrombin-activatable fibrinolysis inhibitor
TM	thrombomodulin
tPA	tissue-type plasminogen activator
TTP	tristetraprolin
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
UTR	untranslated region
VEGF	vascular endothelial growth factor
WT	wild-type
ε-ACA	ε-aminocaproic acid

Chapter 1

General Introduction

1.1 Cancer

Cancer is characterized as a disease of uncontrolled cell growth. It is the leading cause of death in Canada, accounting for 30% of deaths [1]. Additionally, breast cancer is the second leading cause of cancer-related deaths among Canadian women. It is estimated that in 2015, 5,000 Canadian women died from breast cancer. Furthermore, the Canadian Cancer Society estimates that 1 in 9 Canadian women will be diagnosed with breast cancer in their lifetime, making breast cancer the most common type of cancer among Canadian women [1].

Tumorigenesis is a multi-step process involving the accumulation of genetic alternations that results in a malignant phenotype. There are many factors that have been identified that promote tumorigenesis, including environmental factors and familial and genetic influences. Hanahan and Weinberg described six hallmarks of cancer, which are acquired characteristics that are attributed to cancer cells [2, 3]. These hallmarks are alterations to cell physiology that promote tumorigenesis and cancer progression. The six hallmarks of cancer described by Hanahan and Weinberg are: (i) sustaining proliferative signals; (ii) resisting cell death; (iii) evading growth suppressors; (iv) enabling replicative immortality; (v) activating invasion and metastasis; and (vi) inducing angiogenesis [2, 3]. Enhanced understanding of cancer biology has resulted in the proposal of additional hallmarks of cancer, namely contributions from the immune system, as well as interactions with the tumour stroma [4, 5]. The majority of cancer-related deaths can be attributed to metastasis of cancer [6]. Metastasis arises, in part, by activation of cell invasion and

promotion of angiogenesis. Targeting these events may prove to be a beneficial therapeutic strategy to prevent the metastatic outcomes of the disease.

1.1.1 Cancer Metastasis

Cancer metastasis is responsible for approximately 90% of cancer-related deaths [7]. Importantly, it is also the main cause of death in patients with breast cancer. Metastasis is described as the spread of cancer from the primary tumour site to a secondary location within the body. Many challenges exist when targeting metastasis and there are currently no effective therapies to target this process. Therapeutic targeting of metastatic cancers is problematic due to heterogeneity of metastatic lesions, tumour dormancy and drug resistance [8, 9]. It is therefore vital to establish effective therapies that inhibit metastatic growth and also therapies that prevent the metastatic process entirely.

The metastatic cascade is a complex multi-step process that involves: local invasion; intravasation into the blood stream; survival in the circulation; extravasation; and colonization of a distinct site [7]. Success at each step within the cascade is vital for the ultimate goal of cancer metastasis.

Local invasion of cancer is facilitated by physiological processes including extracellular proteolysis, cell migration and epithelial to mesenchymal transition (EMT) [10]. Cell invasion is dependent on interactions in the tumour microenvironment between the cancer cells, basement membrane and stromal cells [11]. The basement membrane is a specialized extracellular matrix (ECM) that separates epithelial tissue from the stromal surroundings. It is composed mainly of type IV collagen, laminins, and fibronectin, with minor contributions from proteoglycans [12]. Physical properties of the ECM can promote

or hinder cell migration and regulation of this dynamic structure is important for cancer progression [13].

Extracellular proteolysis is responsible for degradation of the ECM and basement membrane, which provides a path for invading cancer cells to enter the stroma and ultimately the circulation [14]. In normal tissue, extracellular proteolysis is a highly controlled process; this process, however, is dysregulated in most tumour microenvironments due, in part, to increased expression of matrix-degrading proteases in cancers that proteolytically digest ECM components [15]. There are a large number of proteases that facilitate degradation of the basement membrane. This includes serine proteases, such as plasmin and cathepsin G, matrix metalloproteinases (MMPs), and cysteine proteases, namely cathepsins B and L [13]. Degradation of ECM is predominantly mediated by plasmin and MMPs (see Section 1.3.2). Additionally, degradation of the ECM releases sequestered growth factors that were trapped within the structure during ECM assembly [14].

Cancer cells often acquire a biological program known as EMT, which promotes local invasion and metastasis [16]. EMT is a complex cellular program in which epithelial cells lose their adherent phenotype and cell polarity. This process is regulated by cell signaling, mainly through TGF-β, Notch and Wnt, which results in the downregulation of epithelial-associated genes (i.e. E-cadherin) and upregulation of mesenchymal-associated genes (i.e. N-cadherin) [17-19]. Changes in gene expression of cadherins, integrins and cytoskeletal proteins result in loss of cell-cell attachments and down-regulation of cell adhesion molecules. This leads to loss of epithelial organization and acquisition of a mesenchymal phenotype in which cancer cells detach from adjacent epithelial cells and

basement membrane, which promotes cell motility [16]. EMT in combination with enhanced proteolytic activity, promotes cancer cell migration and invasion into the surrounding stroma.

Intravasation is the process by which invading cancer cells enter the lymphatic system or bloodstream [7]. While this process requires crosstalk between the cancer cells and the tumour microenvironment, the underlying mechanisms are not fully understood. In order for intravasation to occur, cancer cells must cross the endothelial layer by transendothelial migration. This process is thought to occur in two ways. The cancer cells can transmigrate through endothelial cell junctions (paracellular) or can cross through the endothelial cell body (transcellular) [20]. Cytokines and chemokines, secreted from the tumour and stromal cells, increase the permeability of the endothelium, promoting transmigration. Specifically, transmigration through endothelial cell junctions can be accomplished through signalling by TGF-β, TNF-α and Notch, which result in remodelling of endothelial junctions [21-23]. Proteolysis events mediated by MMPs also facilitate endothelial junction remodelling, which also contributes to cancer cell transmigration [24]. Transcellular intravasation involves the formation of a pore through cytoskeletal and membrane remodelling, which allows the cancer cell to cross through the endothelial cell [25, 26]. Although this route is described in literature, it is thought that transendothelial migration through cell junctions is the more frequent method of intravasation [20].

Once the cancer cells have intravasated into the bloodstream, circulating tumour cells (CTCs) must be able to survive a number of stressors encountered in the circulation. This includes forces of shear stress, the presence of immune cells, and induction of anoikis [27]. CTCs are required to withstand shear stress caused by the flowing blood; platelets aid

in this task by associating with the CTCs [28]. Additionally, association with platelets protects CTCs from immune cells found in the bloodstream [28]. In the absence of cell adhesion, epithelial cells normally undergo anoikis, which is a form of programmed cell death triggered by loss of anchorage [7]. In order for CTCs to survive the circulation, they must evade anoikis. CTCs are able to escape anoikis by suppressing cell death signals [29]. Additionally, CTCs that have undergone EMT are more likely to survive the circulation, as the mesenchymal phenotype allows these cells to evade cell death [30].

Surviving CTCs arrest in the capillary bed at a distinct organ site. This is facilitated in part by platelets, which promote cancer cell adhesion to endothelial cells [31]. The cancer cells must then exit the bloodstream or lymphatic system, through a process known as extravasation. It is not expected that mechanisms that drive intravasation are identical to those driving extravasation, due to stromal contributions during the process of intravasation [7]. The factors that drive extravasation are dependent on the site of dissemination. For example, studies have demonstrated that factors that promote extravasation of breast cancer cells in the lungs are distinct from factors that influence extravasation in the bone [32]. Padua *et al.* identified that angiopoietin-like 4 increased breast cancer transendothelial migration to promote extravasation through lung capillaries [33]. Additionally, factors such as COX2, MMP-1 and MMP-2 have been shown to promote pulmonary vascular permeability and extravasation to the lungs [34]. A study by Kang and coworkers demonstrated that a gene expression signature, including IL-11, CTGF, CXCR4 and MMP-1, promotes breast cancer metastasis to the bone [35].

Following extravasation, the cancer cells must survive in the foreign microenvironment and subsequently colonize. Metastatic sites differ depending on the

origin of the primary tumour, and therefore it proposed that microenvironment of the metastatic site is vital for colonization. The seed and soil hypothesis speaks to this concept, stating that the cancer cells are the “seeds” and the foreign organ is the “soil” and the two must be compatible for colonization to occur [36]. The metastatic niche model builds on this hypothesis, emphasizing the importance of the metastatic microenvironment in facilitating colonization at the secondary site [37]. Others argue that cancer cells are more important determinants of colonization then the contributions of the metastatic microenvironment [38]. For colonization to occur, cells entering the new organ must become resistant to the host organ defences to survive [9]. Disseminated cells often survive in a state of dormancy which is thought to be induced by the new host microenvironment, however little is known about the underlying mechanisms responsible for this phenomenon [39]. Disseminated cells must not only survive this dormant state, but also facilitate metastatic outgrowth by reinitiating tumour growth and proliferation [9]. Ultimately, colonization is highly complex and further studies are necessary to delineate factors influencing contributions of the cancer cells and metastatic microenvironment in this process.

Understanding mechanisms that promote the metastatic cascade can aid in the development of therapeutic strategies to combat this phenomenon. Theoretically, there are a number of factors that can be targeted in the metastatic cascade. Current therapies target growth of the primary tumour and have limited impact on the metastatic lesion. Extracellular proteolysis plays a vital in the initial stages of the metastatic cascade and also promotes metastasis at later stages. Therefore, targeting proteolytic events may be a beneficial therapeutic strategy. Several clinical trials attempted to target the proteolytic

activity of MMPs, using MMP inhibitors [40]. However, these trials failed due to side-effects of the inhibitors. These inhibitors were broad spectrum with resulted in off-target effects [41]. These trials were also conducted in patients at advanced stages of the disease, while pre-clinical studies were initiated in animals at early stages [42]. These studies were conducted in patients with established metastatic lesions and therefore targeting initial stages of metastasis or studying preventative therapeutics were not possible in these clinical trials. Additionally, there is redundancy among proteases in the tumour microenvironment and therefore it may be beneficial to target proteases upstream of MMPs.

1.2 Tumour Angiogenesis

Hanahan and Weinberg described sustained angiogenesis as one of the six hallmarks of cancer [2, 3]. Angiogenesis is the sprouting of new vessels from pre-existing blood vessels. Vascular remodelling, through angiogenesis, is an important step in cancer progression, as it provides the tumour with necessary nutrients and also provides an escape route for metastatic tumours. An imbalance of angiogenic inhibitors and activators can stimulate activation of the angiogenic switch, which is point at which tumours transition from an avascular phase to promote vascularization. Most tumours begin to grow in an avascular phase, in which they reach a steady-state of proliferation and apoptosis. In the tumour microenvironment, the angiogenic switch in endothelial cells is triggered when the tumour growth results in hypoxia and nutrient deprivation [43]. As a consequence, the effects of angiogenesis activators, such as vascular endothelial growth factor (VEGF), come to outweigh those of inhibitors, such as thrombospondin-1. Tumours induce angiogenesis by secreting angiogenic activators, such as VEGF and bFGF. Release of angiogenic activators stimulates perivascular detachment and vessel dilation [44]. It also

results in the activation of endothelial cells. Leaky vessels allow plasma proteins to form a matrix to aid in endothelial cell migration [44]. Basement membrane and ECM degradation facilitate endothelial cell migration into the stromal surroundings [45]. Angiogenic factors secreted from the tumour and stromal cells stimulate endothelial cell proliferation and vessel sprouting through cell migration. Perivascular cells are attracted to the sprouting vessels, facilitating vascular basal lamina formation around the new vessel [46].

In addition to playing a pivotal role in the metastatic cascade, extracellular proteolysis promotes angiogenesis by release of sequestered growth factors and by facilitating cell migration [14]. Proteases such as MMPs and plasmin are able to cleave matrix components, which results in the release of sequestered VEGF, which further stimulates the angiogenic switch. Degradation of the ECM also provides a path for proliferating endothelial cells to sprout into new vessels [44].

Blood vessels formed through tumour angiogenesis are irregular. They are morphologically distinct from normal blood vessels and often contain irregular branching patterns [44]. Perivascular cells are often poorly associated with newly formed vessels, resulting in increased vascular permeability. Blood within the tumour vasculature often flows bidirectionally and at a much slower rate than in normal blood vessels [47]. Irregular blood vessel formation results in increased angiogenic activators emanating from leaky vessels [46]. Angiogenic signals in the tumour microenvironment allow for constant growth of blood vessels and prevent endothelial cell quiescence [44].

Tumour angiogenesis is important to the metastatic process. It provides the tumour cells with an escape route. The irregular shape and increased permeability of the tumour vasculature facilitates the intravasation process [7]. Additionally, angiogenesis is pivotal

to the colonization step of metastasis [7]. Activation of the angiogenic switch during colonization allows to the metastatic lesion to transition from the avascular phase to promote vascularization [48], which is essential for growth of micrometastases [49].

1.3 Plasminogen Activation System

Plasminogen is a 791-amino acid plasma zymogen secreted by the liver [50, 51]. It circulates at an approximate concentration of 2 μM [52]. Plasminogen consists of an amino-terminal tail domain, five kringle domains, and a carboxyl-terminal trypsin-like latent serine protease domain [53]. Native plasminogen has an amino-terminal glutamic acid and is therefore referred to as Glu-plasminogen. Glu-plasminogen has a closed tight conformation making it less accessible for activation [54]. Cleavage of Glu-plasminogen at its Lys77-Lys78 bond by plasmin results in the formation of Lys-plasminogen which has a more open conformation and is more susceptible to plasminogen activators (Fig. 1.1) [55]. Cleavage of plasminogen to form plasmin results in a heavy chain consisting of the kringles, disulfide-bonded to the light chain consisting of the protease domain.

There are two physiological plasminogen activators, urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). uPA mediates plasminogen activation in the pericellular context, while tPA mediates plasminogen activation in the context of fibrin [56]. uPA is secreted as a zymogen, pro-uPA, and becomes active following proteolytic cleavage by plasmin. Activation of pro-uPA to uPA by plasmin is enhanced on the cell surface when pro-uPA is bound to its receptor urokinase plasminogen activator receptor (uPAR) [57-59]. tPA is secreted as a single-chain protein, that is proteolytically active. Cleavage of single-chain tPA by plasmin yields two-chain tPA,

which exhibits 50-fold enhanced activity when compared to single chain tPA [60]. These plasminogen activators are inhibited by two members of the serine protease inhibitor superfamily (SERPINS), namely plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2) [56].

1.3.1 Plasminogen Receptors

Plasminogen activation is under tight control. Plasmin formation does not occur at a physiologically-relevant rate in the absence of either fibrin or certain cell surfaces. Accordingly, plasmin activity is only elaborated where it is required. The presence of excess concentrations of SERPINs such as antiplasmin and α_2-macroglobulin ensure that plasmin activity is not disseminated systemically. In the tumour microenvironment, cell surface plasminogen activation likely plays a vital role. Indeed, markers of plasminogen activation on the cell surface are prognostic for cancer progression and metastasis (*vide infra*).

Plasminogen receptors play a vital role in activation of plasminogen to plasmin on the cell surface. They are responsible for localization of plasminogen activation, which is important in mediating plasmin-dependent processes. Plasminogen receptors are also important in accelerating plasminogen activation on the cell surface and protecting plasmin from its natural inhibitor, antiplasmin [61]. Plasminogen receptors are found on a range of cell types, including endothelial cells, monocytes, macrophages and cancer cells [62-64]. Plasminogen binds to plasminogen receptors most commonly through lysine binding sites within its kringle domains [65].

The majority of plasminogen receptors mediate their interaction with plasminogen through lysine binding (Fig. 1.1). Based on the characteristic of lysine binding, there are

four classes of plasminogen receptors [60, 62]. The first class contains a carboxyl-terminal lysine residue upon synthesis and promotes plasminogen activation. The second class lacks a carboxyl-terminal lysine residue when synthesized, but upon proteolytic cleavage a carboxyl-terminal lysine becomes exposed. The third class of plasminogen receptors do not possess either a carboxyl-terminal lysine residue or internal lysine residue that can be exposed, and yet can still accelerate plasminogen activation. The fourth class of receptors are molecules that bind to plasminogen but do not promote plasminogen activation [60, 62].

The majority of plasminogen receptors possess a carboxyl-terminal lysine or an internal lysine residue that can be exposed upon cleavage. The importance of lysine binding in plasminogen activation has been demonstrated using lysine analogs and basic carboxypeptidase activity. Binding of lysine analogs to plasminogen kringles block plasminogen binding to the cell surface [66]. Lysine analogs have also been shown to allow Glu-plasminogen to adopt a more open conformation, making it more accessible to plasminogen activators in solution [67]. Additionally, studies have shown that carboxypeptidase B can inhibit plasminogen activation by cleaving carboxyl-terminal lysine residues from plasminogen receptors [68].

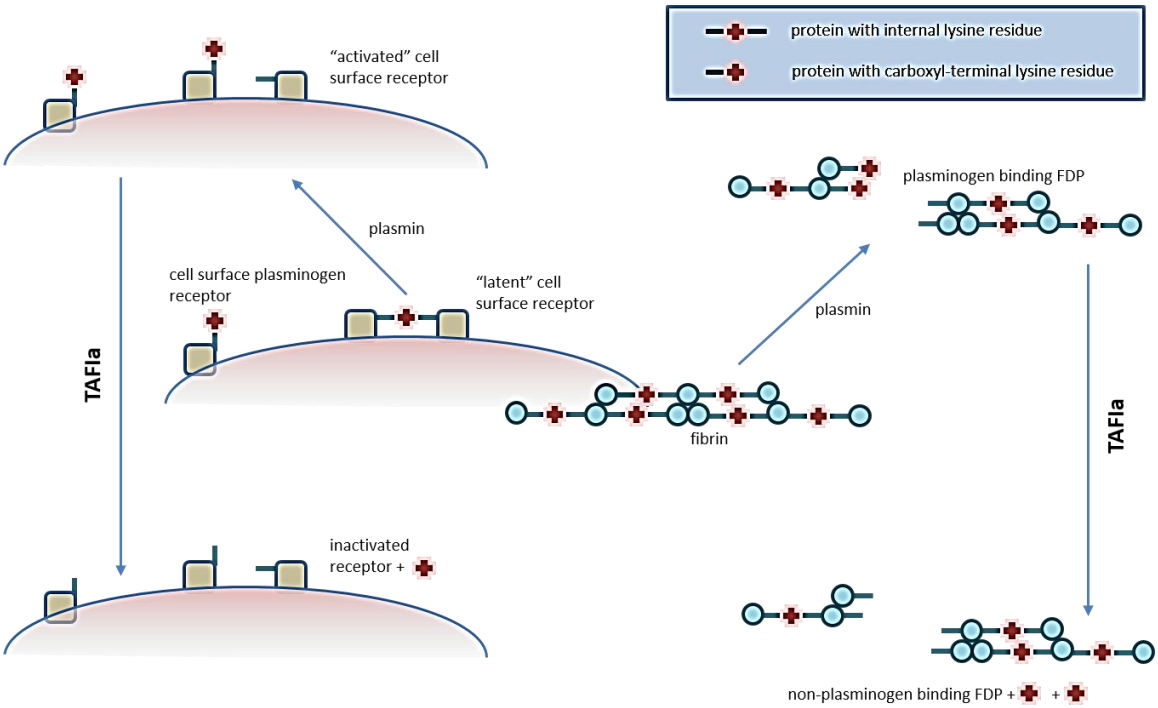

Figure 1.1: Carboxyl-terminal lysine residues in plasminogen activation on the cell surface and on fibrin.
Cell surface plasminogen receptors can possess a carboxyl-terminal lysine residue (shown in red) or an internal lysine residue that can be cleaved to expose a carboxyl-terminal lysine residue. These residues allow for plasminogen binding to the cell surface and accelerate plasminogen activation to plasmin. TAFIa cleaves carboxyl-terminal lysine residues from plasminogen receptors on the cell surface, attenuating plasminogen activation. When fibrin is cleaved into fibrin degradation products (FDPs) by plasmin, carboxyl-terminal lysine residues on FDPs are exposed. Plasminogen is able to bind to FDPs, accelerating plasminogen activation. TAFIa cleaves carboxyl-terminal lysine residues from FDPs, preventing plasminogen binding and activation. Adapted from reference [69].

Plasminogen receptors have been shown to enhance cell surface plasminogen activation, by accelerating conversion of Glu-plasminogen to Lys-plasminogen. Binding of Glu-plasminogen to plasminogen receptors results in a conformational change that exposes the plasmin cleavage site on plasminogen, promoting the formation of Lys-plasminogen [70-72]. Plasmin generation is dependent on a positive feedback mechanism that allows for accelerated plasminogen activation. Glu-plasminogen is activated to Glu-plasmin by proteolytic cleavage at Arg561-Val562 by plasminogen activators (Fig. 1.2) [73]. The small amount of Glu-plasmin generated is able to accelerate further activation of plasminogen, by activation of plasminogen activator and conversion of Glu-plasminogen to Lys-plasminogen. Plasmin cleaves Glu-plasminogen at Lys77-Lys78 resulting in Lys-plasminogen, which has a more open conformation and is more susceptible to plasminogen activators. Lys-plasminogen is subsequently proteolytically cleaved at Arg561-Val562, by plasminogen activators resulting in the generation of Lys-plasmin [73]. Plasmin is a broad-spectrum specificity serine protease, as it has been shown to cleave a large number of substrates. Plasmin is important for physiological and pathological functions, including fibrinolysis, inflammation, angiogenesis, wound healing and cancer metastasis [52, 62, 74].

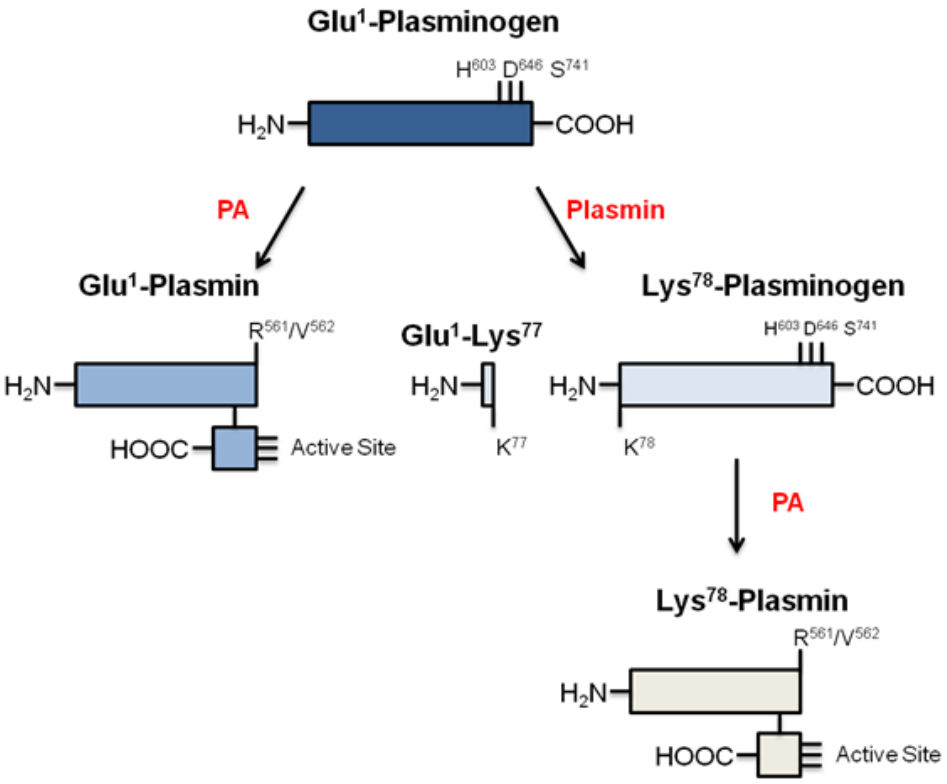

Figure 1.2: Activation of Plasminogen to Plasmin.
Native plasminogen circulates as a zymogen with an amino-terminal glutamic acid. Glu-plasminogen is converted to Glu-plasmin through proteolytic cleavage at Arg561, by plasminogen activators (PA), exposing the serine protease active site. Plasmin cleaves Glu-plasminogen at Lys77, generating Lys-plasminogen. Lys-plasminogen has a more open conformation and is more susceptible to cleavage by plasminogen activators. Lys-plasminogen is cleaved by plasminogen activators at Arg561 to produce Lys-plasmin. Used with permission from reference [75].

1.3.2 Plasminogen Activation System in Metastasis and Angiogenesis

The plasminogen activation system has a well-established role in cancer progression and metastasis. Factors of the plasminogen activation system are overexpressed in most cancers and their expression frequently correlates with poor prognosis. Expression of plasminogen receptors is correlated with invasive breast cancer [76, 77]. Additionally, elevated levels of plasminogen receptors in breast cancer is associated with decreased survival [77-79]. Increased uPAR levels in breast cancer is an independent prognostic marker of shortened relapse-free survival [80-82]. Levels of uPA and PAI-1 are also prognostic markers in breast cancer and uPA may be an independent variable used to determine recurrence [83-85].

Plasminogen activation system is involved in several functions related to cancer, including cell adhesion, cell migration, proliferation, angiogenesis and metastasis [74, 86]. Activation of plasminogen to plasmin promotes metastasis through several mechanisms. Plasmin itself cleaves extracellular matrix (ECM) components, such as fibronectin, vitronectin, laminin and collagen, facilitating degradation of the ECM, cell invasion and ultimately metastasis (Fig. 1.3) [87-89]. Plasmin also activates other proteases and growth factors that promote ECM degradation, tumour growth and angiogenesis. Specifically, *in vitro* plasmin can activate many MMPs, including MMP-1, 2, 3, 9, 13 and 14 [52]. Activation of MMPs in the tumour microenvironment have been shown to be vital in cancer metastasis and angiogenesis, specifically through ECM degradation, growth factor activation and cell signalling [74, 90].

By degrading the ECM, plasmin also releases sequestered growth factors, whose downstream targets facilitate tumour progression. Studies, both *in vitro* and *in vivo*, have

demonstrated that plasmin can liberate basic fibroblast growth factor (bFGF) [91, 92]. This growth factor mediates cellular processes such as cell proliferation, and studies have demonstrated its involvement in angiogenesis [93, 94]. Studies have also shown that bFGF can increase secretion of uPA, suggesting a possible role in mediating positive feedback for plasminogen activation [95]. Furthermore, release and activation of sequestered VEGF by plasmin and MMPs stimulates angiogenesis in the tumour microenvironment [74]. Plasmin is also responsible for proteolytic activation of latent TGF-β1 to its active form, which mediates cancer cell invasion and metastasis through promotion of EMT [96]. Studies have also demonstrated that intravasation is more effective when cancer cells have high levels of uPAR and uPA and inhibition of plasmin hindered the process of intravasation [97, 98].

The plasminogen activation system is also able to promote cancer cell invasion and metastasis through cell signalling by uPAR. Interaction of uPA to uPAR mediates activation of various signalling pathways that influence cell motility [99]. uPAR does not possess an intracellular domain and therefore uPAR-mediated intracellular signalling results from interaction with various integrin and ECM proteins [100]. Overexpression of uPAR and binding of uPA to its receptor promotes cell migration and adhesion through binding with integrins and ECM proteins [99]. Specifically, studies have demonstrated that binding of uPA to uPAR stimulates cell invasion and migration by cell signalling through Ras/Raf/MEK/ERK pathway [101].

Inhibition of plasminogen activation, by targeting plasminogen activators or plasminogen receptors, has been thought to be viable therapeutic strategy to inhibit the effects of the plasminogen activation system on metastasis [62, 102, 103]. Specifically,

carboxyl-terminal lysine residues on plasminogen receptors are an attractive target, given the importance of these residues in plasminogen activation on the cell surface. Targeting of the plasminogen activation system would impact proteolytic events downstream of plasmin formation.

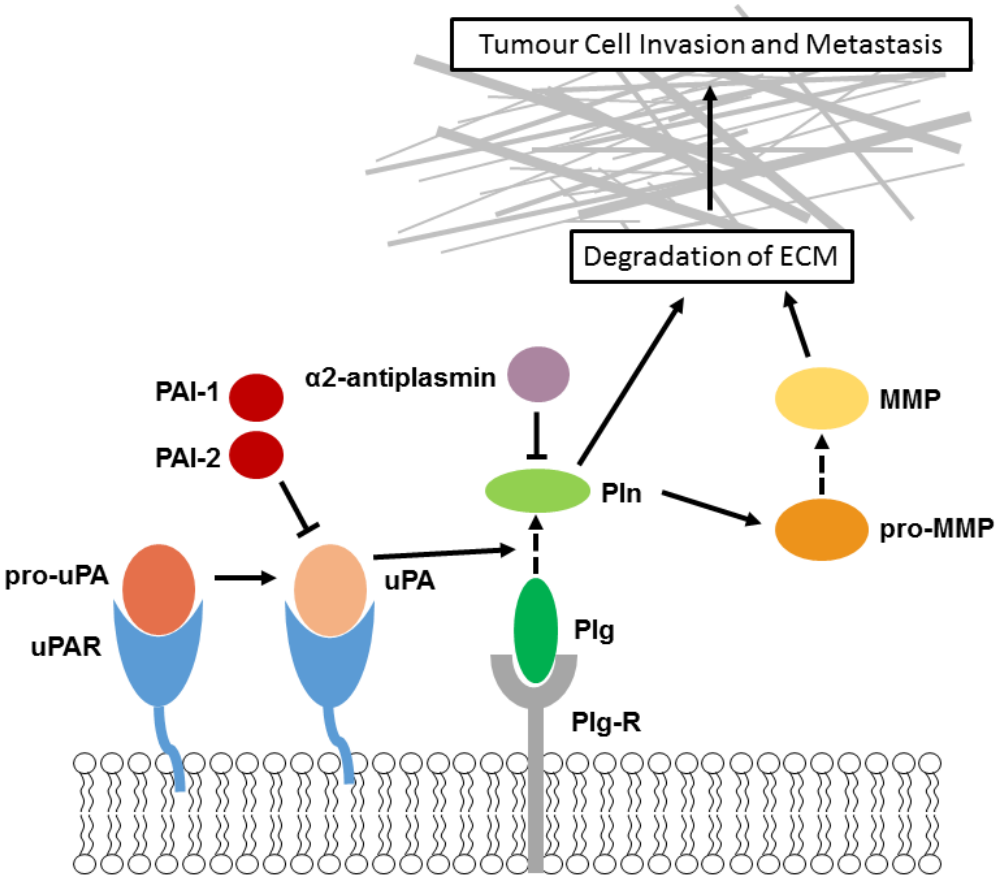

Figure 1.3: Plasminogen activation system in cancer.
Pro-uPA binds to uPAR on the cell surface promoting activation to active uPA through cleavage by plasmin. Plasminogen (Plg) binds to its cell surface plasminogen receptor (Plg-R) which accelerates uPA-mediated Plg activation to plasmin (Pln). Pln activates pro-MMPs to their active MMP form and together Pln and MMPs degrade the ECM, facilitating tumour cell invasion and metastasis. This system can be inhibited by PAI-1 and PAI-2, which inhibit uPA and α2-antiplasmin, which inhibits Pln.

1.4 Thrombomodulin (TM)

Thrombomodulin (TM) is 557-amino acid long, single-chain type 1 transmembrane protein [104]. It is found on the surface of endothelial cells, monocytes, smooth muscle cells, neutrophils and is also expressed in cancer cells, including breast [105-109]. TM is comprised of five domains, as shown in Figure 1.4. The extracellular domains of TM include an amino-terminal C-type lectin-like domain, six epidermal growth factor (EGF)-like repeats, and a serine/threonine rich domain [104]. TM also contains a transmembrane domain and a short cytoplasmic tail. This multifaceted glycoprotein is involved in vital physiological functions such as inflammation and coagulation, and studies have also demonstrated its involvement in cancer biology [110-112].

The C-type lectin-like domain of TM is 222 amino acids in length and has homology with other C-type lectins. However, the lectin-like domain of TM does not contain a calcium binding motif unlike traditional C-type lectins [113, 114]. Additionally, while conventional C-type lectins bind only to carbohydrates, the lectin-like domain of TM can also bind protein ligands. Binding of the C-type lectin-like domain of TM to several ligands mediates inflammation and cell adhesion [115, 116]. The first evidence of the anti-inflammatory role of TM was demonstrated by generating mice lacking the lectin-like domain of TM [115]. These mice were more susceptible to LPS-induced sepsis. It was later determined that the lectin-like domain of TM can bind the carbohydrate Lewis Y antigen in LPS found on gram-negative bacteria [117]. Binding to TM prevents LPS binding to CD14, and therefore decreases the inflammatory response stimulated by LPS. The anti-inflammatory effects of TM were further demonstrated when Abeyama *et al.* reported that

binding of high mobility group box 1 (HMGB1) protein to the lectin-like domain of TM sequesters HMGB1, preventing it from eliciting a pro-inflammatory response [118].

The EGF-like domain is responsible for the thrombin cofactor activity of TM. Thrombin binds to EGF-5 and EGF-6 of TM and this interaction mediates the activation of both thrombin-activatable fibrinolysis inhibitor (TAFI) and protein C (PC) by thrombin [119, 120]. Specifically, in the presence of TM, catalytic efficiencies of thrombin-mediated activation of both TAFI and PC are enhanced by over 1000-fold [121, 122]. Importantly, domain deletions have identified essential domains necessary for TAFI and PC activation by thrombin/TM. Specifically, the c-loop of EGF-3 through EGF-6 are required for TAFI activation, while the interdomain loop connecting EGF-3 to EGF-4 plus EGF4-6 are required for PC activation. Using site-directed mutagenesis, Wang and coworkers identified residues within the EGF-like domain of TM that are important for activation of TAFI and PC by thrombin [123]. Amino acids Val340 and Asp341 within the c-loop of EGF-3 of TM were found to be vital for activation of TAFI. Amino acid Phe376 in EGF-4 was reported to be necessary for protein C activation. Glaser *et al.* reported that TM can be oxidized at Met388 in the linker region between EGF-4 and EGF-5 [124]. Interestingly, oxidation of Met388 drastically reduces thrombin/TM mediated activation of PC, but does not impact activation of TAFI [123]. This may act as a potential mechanism to support preferential activation of TAFI over PC at the site of injury, in which neutrophils would promote TM oxidation [108, 125]. Furthermore, mutagenesis of Gln387 to a proline abolishes thrombin binding to TM, and therefore results in significant reduction of both TAFI and protein C activation [123].

The anti-coagulation function of TM is mediated through binding with thrombin. Interaction with TM inhibits the pro-coagulant functions of thrombin, by altering its substrate specificity. This results in inhibition of anion-binding exosite-mediated thrombin binding to fibrinogen, preventing cleavage of fibrin [122], while promoting activation of protein C to activated protein C (APC). Structural studies suggest that TM binds to PC, presenting it optimally as a substrate for thrombin in the ternary complex [126]. APC possesses anti-coagulant activity by cleaving activated coagulation factors Va and VIIIa, which attenuates further generation of thrombin [122, 127, 128]. Paradoxically, TM also acts as an anti-fibrinolytic molecule, through activation of TAFI. As previously mentioned, the thrombin/TM complex activates TAFI to its active form activated TAFI (TAFIa), which possesses basic carboxypeptidase activity [121]. TAFIa attenuates fibrinolysis by removing carboxyl-terminal residues from partially degraded fibrin [129]. These residues act as cofactors for plasminogen activation to plasmin during fibrinolysis, and are important in several positive feedback mechanisms in fibrinolysis. The anti-coagulant and anti-fibrinolytic activities of TM are dependent on not only TM concentrations but also on complex interplay between coagulation and fibrinolysis factors. Specifically, concentrations of activators, inhibitors and cofactors involved in coagulation and fibrinolysis contribute to the anti-coagulant and anti-fibrinolytic functions of TM.

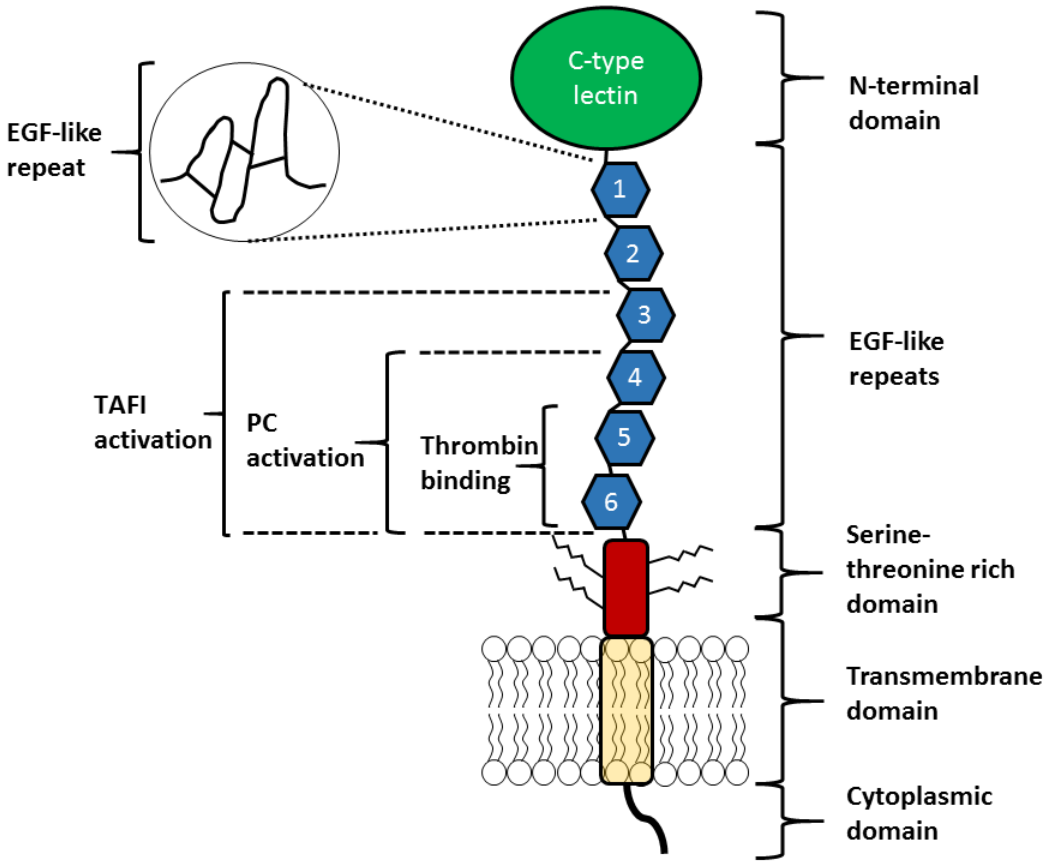

Figure 1.4: Structure of Thrombomodulin.
Thrombomodulin contains a C-type lectin-like domain, six EGF-like repeats, a serine/threonine rich domain, a transmembrane domain and cytoplasmic tail. The structure of an EGF-like repeat is shown, containing three disulfide bonds within the three loop structure. The EGF-like domain is responsible for the cofactor functions of TM, involving thrombin-mediated activation of TAFI and protein C. EGF5-6 bind to thrombin, EGF3-6 are necessary for activation of TAFI and EGF4-6 are necessary for activation of protein C.

1.4.1 Thrombomodulin in Cancer and Angiogenesis

TM is expressed in several cancers, including breast, bladder, cervical, ovarian, colorectal, melanoma, and prostate [109, 130-136]. Loss of TM expression is associated with advanced disease stage and metastatic potential. Studies in melanoma and prostate cancers have demonstrated that loss of TM expression is a result of hypermethylation of the *THBD* promoter region [135, 136]. Furthermore, loss of TM has been associated with poor prognosis in several cancers. Specifically, TM expression was found to be significantly inversely correlated to survival in non-small cell lung cancer [137]. In hepatocellular carcinoma, tumours positive for TM expression were associated with higher recurrence-free survival compared to tumours negative for TM [138]. This study also found that TM-positive tumours had lower frequencies of intrahepatic metastasis. In addition, Hamatake *et al.* reported loss of TM expression in lung squamous cell carcinoma was associated with decreased survival in patients [139]. They also found decreased TM expression in metastatic lesions compared to primary tumours. In breast cancer patients, low TM expression was correlated with high relapse rates [109]. In bladder cancer, decreased TM expression was found to be correlated with advanced clinical stage [130]. Therefore, the evidence is consistent with the idea that TM is an anti-metastatic factor, although mechanistic studies are required to demonstrate a direct role for this factor.

Indeed, researchers have sought to understand the role of TM in regulating metastatic potential. Specifically, a study using murine melanoma cells indicated that TM is capable of inhibiting cell invasion *in vitro* and *in vivo* [134]. Furthermore, overexpression of TM suppressed migration of both cervical and ovarian cancer cells *in vitro* [131, 132]. Wu and coworkers reported that TM mediates migratory ability of prostate cancer cells by

regulating epithelial to mesenchymal transition (EMT) [140]. Additionally, silencing of TM increased invasiveness of bladder cancer, both *in vitro* and *in vivo*. Decreased TM in bladder cancer also resulted in increased tumour growth, angiogenesis and EMT. The mechanisms by which TM suppresses cancer cell invasion and migration are not fully understood, however several studies have demonstrated an important role for TM in mediating EMT. Specifically, a study found that TM expression is inversely correlated to Snail expression in tumour cells [141]. Furthermore, the same study found that decreased TM expression inhibits trafficking of E-cadherin to the cell membrane [141]. Recently, Zheng *et al*. reported that thrombomodulin reduces metastatic potential of lung cancer cells by up-regulating E-cadherin expression [142]. This is consistent with studies in cervical, prostate and liver cancer, in which down-regulation of TM decreased E-cadherin expression [131, 140, 143].

Recently, Horowitz and coworkers demonstrated the importance of the thrombin-binding domain of TM on metastasis *in vivo* [144]. Lack of thrombin binding to TM resulted in a metastatic phenotype in nude mice. This indicates a potential role for the thrombin/TM complex in mediating the anti-metastatic effects of TM. Given that the thrombin/TM complex is responsible for the activation of TAFI and protein C, this result suggested a role for one or both of these thrombin/TM substrates in mediating the anti-metastatic effects of TM.

Thrombomodulin has been shown to be both pro- and anti-angiogenic. Shi and coworkers reported that a variant of TM that only included the EGF-like domain and the serine/threonine rich domain enhanced angiogenic responses, *in vitro* and *in vivo* [145]. This was followed up with a more recent study that determined that this variant of TM

induced angiogenesis by activating Type 1 fibroblast growth factor receptor (FGFR1) [146]. Using site-directed mutagenesis, this study reported that the effects of TM are independent of its ability to activate protein C. Conversely, the same group found that the C-type lectin-like domain of TM acts as an anti-angiogenic factor, by interacting with Lewis Y antigen [147]. This indicates that depending on the stimulus, TM may act as both a pro- and anti-angiogenic factor.

1.5 Thrombin-Activatable Fibrinolysis Inhibitor (TAFI)

Since 1989, TAFI has been discovered independently by several different groups, based on various characteristics. In 1989, Hendricks *et al.* described basic carboxypeptidase activity in serum that disappeared following 2-hour incubation at 37°C [148]. It was determined that this carboxypeptidase activity was unrelated to the constitutively active plasma carboxypeptidase N. Due to the instability of this newly described carboxypeptidase, it was named “unstable” carboxypeptidase (or CPU). Also in 1989, in an independent study, Campbell and Okada identified a basic carboxypeptidase in serum that cleaved carboxyl-terminal arginine residues, preferentially over lysine [149]. Therefore, they it was given the name arginine carboxypeptidase (or CPR). In 1991, a plasminogen binding protein was isolated from plasma and named procarboxypeptidase B (proCPB) because cDNA sequence analysis revealed this protein is homologous to pancreatic carboxypeptidase B [150]. In 1995, Bajzar and coworkers purified a plasma zymogen that when activated by thrombin, inhibits fibrinolysis. Therefore, they named this zymogen TAFI [151]. Amino acid sequencing analysis revealed that CPU, CPR, proCPB and TAFI are identical proteins [150-152].

Since its initial discovery, the role of TAFI in attenuating fibrinolysis has been well established. TAFI, when activated to its active form, TAFIa, cleaves carboxyl-terminal lysine residues from partially degraded fibrin. These lysine residues act as plasminogen and tPA binding sites on fibrin, which accelerate plasmin generation and ultimately fibrinolysis. By removing these lysine residues, TAFIa is able to inhibit plasmin formation and attenuate fibrinolysis. In addition to inhibiting plasminogen activation in the context of fibrinolysis, TAFIa has also been shown to inhibit cell surface plasminogen activation, through cleavage of plasminogen receptors [69].

1.5.1 CPB2 Gene Expression and Regulation

CPB2 is the gene encoding TAFI, as designated by the Human Genome Organization (HUGO), and is located on chromosome 13 (specifically 13q14.11) [152]. The gene contains 11 exons and 10 introns, spanning 48 kilobase pairs of genomic DNA [153]. There have been no genetic disorders identified in association with *CPB2*. *CPB2* is mainly transcribed in the liver, however extra-hepatic expression of *CPB2* has been observed. Specifically, *CPB2* mRNA has been detected in platelet α-granules, megakaryocytes, monocytes, monocyte-derived macrophages, adipocytes and endothelial cells [154-157]. However, our study, using an appropriate method of TAFI detection, failed to yield evidence of TAFI protein in many of these sites [157]. In our study TAFI protein was detected in macrophages and megakaryocytes.

Plasma TAFI concentrations vary within the population from approximately 75 to 275 nM; however, genetic factors such as SNPs in *CPB2* only account for approximately 25% of the variability [158, 159]. It is thus thought that the majority of variation associated with plasma TAFI levels arises from gene regulation. Understanding transcriptional and

post-transcriptional regulation of *CPB2* provides important insight into factors influencing plasma TAFI concentrations, in haemostasis, during inflammation and disease states. Regulation of *CPB2* occurs through different molecular mechanism including regulation of gene expression, mRNA stability and alternative splicing of transcripts.

The 5' and 3' flanking regions of *CPB2* have been extensively studied. The 5' flanking region lacks a TATA box and instead transcription is initiated from multiple transcription start sites [153]. This feature is common among some proteins with liver-specific expression. Based on the transcription initiation start site used, 5'-untranslated regions (UTR) of the mRNA transcripts vary in length. Nucleotides -80 to -73 of the 5' flanking region are important for basal hepatic expression, as deletion of these base pairs results in an elimination of promoter activity. Several transcription factor binding sites have been identified in the promoter region of *CPB2*. A CCAAT/enhancer binding protein (C/EBP) site [160] may be important in restricting *CPB2* expression to certain cells. A hepatocyte nuclear factor-1α (HNF-1α) binding site was also found in the promoter region and is important for liver-specific expression of *CPB2* [161]. Furthermore, a nuclear factor-Y (NF-Y) binding site is important for basal promoter activity of *CPB2* [161]. Additionally, a glucocorticoid responsive element (GRE) was identified in the promoter and a synthetic glucocorticoid hormone analog was able to stimulate *CPB2* promoter activity [162]. Glucocorticoid is an important steroid hormone that functions to reduce inflammation, by up-regulating the expression of anti-inflammatory proteins. The presence of a GRE in the *CPB2* promoter indicates a potential role for TAFI in inflammation. Hori and coworkers reported that *CPB2* gene expression in adipocytes is regulated by insulin through

phosphatidylinositol-3-kinase (PI3K)/Akt pathway [156], although others studies called into question whether *CPB2* is truly expressed in those cells [155].

The 3'UTR of *CPB2* plays an important role in mRNA regulation. Analysis of the 3' flanking region of *CPB2* revealed sequences that correspond to three different polyadenylation sites [153]. Use of these sites during mRNA processing results in transcripts with different length 3' UTR, specifically 390, 423 or 549 nucleotides in length [153]. Studies have demonstrated that the stability of the mRNA transcript is dependent the polyadenylation site used [163]. Use of the first polyadenylation site results in the shortest and most stable transcript. As the length of the transcript increases, there is a decrease in stability. Hence, use of the third polyadenylation site results in the longest and least stable transcript.

Studies have identified both *cis*-elements and *trans*-acting factors that modulate transcript stability, polyadenylation site usage and transcript abundance. Inflammatory cytokines interleukin-1β (IL-1β) and IL-6 were found to decrease the stability of the *CPB2* transcript and shift the abundance of the transcript to the longest and least stable [163]. Treatment of IL-1β and IL-6 therefore promote use of the third polyadenylation site. Progressive deletions of nucleotides within the 3' flanking region identified one stability element upstream of the first polyadenylation site, as well as three instability elements, one located upstream of each polyadenylation site. Mobility shift assay identified tristetraprolin (TTP) as a *trans*-acting factor that binds to the third instability element (upstream of the third polyadenylation site) and causes destabilization of the transcript [164]. A recent study determined that TNF-α and lipopolysaccharide (LPS) down-regulated *CPB2* mRNA and TAFI protein levels in HepG2 cells [165]. These pro-inflammatory factors effect mRNA

stability dependent on TTP binding. Furthermore, the same study found that treatment of HepG2 cells with anti-inflammatory cytokine IL-10 increased mRNA stability and abundance, independent of TTP binding [165]. This study also identified HuR as a *trans*-acting factor that binds to the 3' UTR and stabilizes the transcript. Masuda and coworkers reported that the PI3K pathway is important in post-transcriptional regulation of *CPB2* in hepatocytes, as use of a PI3K inhibitor decreased mRNA abundance and stability [166]. Intriguingly, cytokines that influence mRNA regulation in hepatocytes have distinct effects in macrophages [165]. Specifically, pro-inflammatory factors LPS, TNF-α, IL-8 and IL-6 + IL-1β all resulted in an increase in mRNA abundance and stability. These results indicate that post-transcriptional regulation of *CPB2* is tissue-specific.

Several single nucleotide polymorphisms (SNPs) have been identified throughout the *CPB2* gene. Specifically, SNPs have been identified in the 5' flanking region, the coding region the 3' flanking region [167, 168]. In addition to SNPs reported in literature, HapMap and dbSNP databases revealed 72 and 339 SNPs in *CPB2*, respectively [169]. SNPs within the 5' flanking region of *CPB2* were not found to influence promoter activity, and indeed they are present between known transcription factor binding sites. Of the SNPs identified in the coding region, only two result in amino acid substitutions, +505G/A and +1040C/T [168]. These nucleotide changes correspond to Ala147Thr and Thr325Ile, respectively. Three SNPs in the 3' flanking region, +1344 G/A, +1542 C/G and +1583 A/T, all correspond to changes in *CPB2* mRNA stability [167] and thus may be causally related to variation in plasma concentrations of TAFI.

Matsumoto and coworkers identified an alternatively spliced variant of TAFI which was found in the hippocampus [170]. This TAFI variant is translated from a *CPB2*

transcript lacking exon 7 and contains a 52-base pair deletion in exon 11. They reported that this variant of TAFI can cleave β-amyloid precursor protein (APP) and $A\beta_{1-42}$, which is an APP-derived β-amyloid peptide. Further studies identified additional splice variants in various cell types. Cagliani *et al.* reported *CPB2* mRNA lacking exon 7 in HepG2 cells [171]. Exon 7 skipping was also reported in Dami megakaryocytes, THP-1-derived macrophages, peripheral blood mononuclear cells, platelets, testis and cerebellum [172]. Additionally, exon 7 and 8 skipping was detected in liver and megakaryocytes [172]. It was determined that these spliced transcripts of *CPB2* are translated but are retained within the cell instead of being secreted. Alternative splicing of *CPB2* may be a mechanism of tissue specific down-regulation of *CPB2* or may generate intracellular isoforms of TAFI with novel functions.

1.5.2 Variations and Associations in Plasma TAFI Concentrations

Plasma TAFI concentrations have been shown to be influenced by certain disease states. Studies have found an increase in plasma TAFI concentrations in patients with diabetes, renal disease, primary hypothyroidism, Behcet's disease, and polycystic ovary syndrome [173-180]. Studies have demonstrated that obesity also influences plasma TAFI levels, as they have been found to be higher in obese individuals versus non-obese individuals [155]. On the other hand, patients with cirrhosis or inflammatory bowel disease had plasma TAFI concentrations that were less than healthy individuals [181-183]. Clinical studies have also examined plasma TAFI concentrations as a potential risk factor for certain cardiovascular diseases. Specifically, clinical studies have shown that elevated plasma concentrations of TAFI are associated with increased risk for venous thrombosis, venous thromboembolism and ischemic stroke [184-187].

Plasma TAFI levels have been shown to increase with age in women but not men [188, 189]. Additionally, there is an increase in TAFI levels in postmenopausal women when compared to premenopausal women. Plasma TAFI concentrations decreased with administration of selective estrogen receptor modulators, including HMR 3339 and raloxifene [190, 191]. Additionally, there was a decrease in plasma TAFI levels using hormone replacement therapy (HRT), specifically, transdermal or oral estradiol or estradiol in combination with trimegestone [192, 193]. Conversely, studies have found no difference in plasma TAFI concentrations upon administration of HRT and SERMs [194, 195]. A study in HepG2 cells aided in the understanding of the role of hormones in *CPB2* expression and regulation [196]. This study determine that estrogen and progesterone were able to decrease expression of *CPB2*, although the estrogen and progesterone receptors were not found to bind to the *CPB2* promoter. The effects of these hormones were found to be independent of the estrogen receptor and instead dependent on the PI3K pathway [196]. Paradoxically, studies have demonstrated that plasma TAFI concentrations are higher in pregnant women when compared to non-pregnant women [197-199]. Levels of TAFI increased during the gestation period, peaking at 20-39 weeks and promptly decreasing following delivery [197-199]. Therefore, though there is a clear role for sex hormones in regulating plasma TAFI levels, the mechanistic details underlying these observations and their physiological relevance remains unclear.

1.5.3 Structure and Activation of TAFI

The TAFI protein is synthesized as a 423 amino acid pre-proenzyme. It contains a 22 amino acid signal peptide that is cleaved prior to secretion [150]. The mature protein therefore contains 401 amino acids, with a predicted molecular mass of 46 kDa. Due to

glycosylation sites in the amino-terminal domain of the protein, the molecular mass of TAFI when observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is approximately 60 kDa [151]. TAFI contains two domains, the activation peptide (spanning from Phe1-Arg92) and the catalytic domain (Ala93-Val401) [200].

The activation peptide of TAFI contains two parts. Phe1-Val76 form an open sandwich antiparallel α/β-fold with four β-strands and two α-helices [200]. The linker region, from amino acids Glu77-Arg92, is partially α-helical in structure [200]. There are four *N*-linked glycosylation sites within the activation peptide of the protein (at Asn22, Asn51, Asn63 and Asn86) [201]. Glycosylation of the activation peptide allows for proper folding and secretion of the zymogen. The activation peptide is also important in preventing substrates from entering the active site of the zymogen. Furthermore, the activation peptide plays a vital role in restricting movement of the dynamic flap region, prior to activation of TAFI. The dynamic flap is a region of the catalytic domain important for the stability of TAFIa and will be discussed in more detail in section 1.5.4.

TAFI circulates in plasma as the zymogen. It is activated to its active form, TAFIa, by proteolytic cleavage at Arg92 (Fig. 1.5). TAFI is activated by thrombin, plasmin or most effectively by thrombin in complex with TM Thrombin and plasmin are both weak activators of TAFI, therefore large amounts of these enzymes are required for TAFI activation. The endothelial cofactor TM enhances catalytic efficiency of thrombin-mediated TAFI activation by 1,250-fold [121]. Plasmin-mediated activation of TAFI is enhanced approximately 15-fold by glycosaminoglycans [202]. Compared to plasmin in the presence of glycosaminoglycans, TM-dependent activation by thrombin is still 10-fold more efficient [202]. Therefore, the thrombin/TM complex is thought to be a vital

physiological activator of TAFI. Indeed, a baboon model of sepsis identified thrombin/TM as the predominant activator of TAFI in this setting *in vivo* [203]. Mosnier and coworkers determined that, in plasma, thrombin/TM mediated activation of TAFI is optimal at 5 nM TM [204]. Concentrations of TM greater then 5 nM decreased TAFI activation in plasma. This phenomenon was also demonstrated using microvascular endothelial cells [204]. Cell density (corresponding to an increase in TM concentration) was inversely correlated to activation of TAFI. This was not observed in plasma deficient of protein C, indicating competitive binding of these thrombin/TM substrates. Accordingly, it was concluded that at concentrations of TM greater than 5 nM, activation of protein C inhibits thrombin generation and subsequent activation of TAFI. Therefore, *in vivo* activation of TAFI by thrombin/TM depends on the site of activation, as concentrations of TM vary depending on blood vessel size [205].

There have been studies aimed at identifying residues important for TM-dependent TAFI activation. Substitution of three consecutive lysine residues in the activation peptide (Lys42-44) to alanines, resulted in an 8-fold reduction in thrombomodulin-dependent TAFI activation [206]. Additionally, a recent study identified Arg12 and Arg377 of TAFI as important residues in thrombin/TM mediated activation [207]. This is consistent with a study by Plug and coworkers which demonstrated that mutagenesis of Arg12 to Gln significantly decreased thrombin/TM mediated activation of TAFI [208]. It is thought that Arg12 interacts directly with TM, while Arg377 interacts with thrombin in the presence of TM. Additionally, Zhou *et al.* also demonstrated the importance of the activation peptide in thrombomodulin-dependent activation of TAFI [209]. Deletion of amino acids 1-73 of

the activation peptide abolished the cofactor ability of thrombomodulin, with respect to thrombin-mediated activation of TAFI [209].

Once activated, TAFIa possesses basic carboxypeptidase activity, cleaving carboxyl-terminal arginine and lysine residues from protein and peptide substrates. TAFIa is a member of the metallo-carboxypeptidase family, as it contains a Zn^{2+} ion in the active site, which is coordinated by His159, Glu162 and His288 [210, 211]. The Zn^{2+} ion aids in the deprotonation of an incoming water molecule [210]. The key catalytic residue in the active site is Glu363, which is responsible for abstracting the proton from the water molecule [211]. The oxygen of the carbonyl group of scissile peptide bond in the substrate is polarized by the presence of Arg217 in the active site. These residues allow for nucleophilic attack on the carbonyl carbon. Specificity of TAFIa for substrates containing carboxyl-terminal arginine or lysine residues is mediated by the presence of Asp348 at the base of an elongated specificity pocket. The Zn^{2+} ion and Arg217 aid in the stabilization of the tetrahedral intermediate. The tetrahedral intermediate collapses as the nitrogen of the peptide bond of the substrate removes the proton from Glu363. The products are released and the enzyme is able to receive a new substrate [211].

1.5.4 Intrinsic Instability and Inactivation of TAFIa

A distinct characteristic of TAFIa is that it is intrinsically unstable and undergoes temperature-dependent spontaneous decay. TAFIa therefore possesses a short half-life of approximately 8-15 minutes at 37°C [212]. The exact mechanism by which TAFIa spontaneously decays is unknown, however crystal structures have identified a region of the protein linked to its instability [200, 213]. The instability of TAFIa results from a highly mobile region, named the dynamic flap (Phe297-Trp350). This region is stabilized in the

zymogen by hydrophobic interactions with the activation peptide. Specifically, amino acids Val35 and Leu39 within the activation peptide interact with Tyr341 to stabilize the dynamic flap [200]. When TAFI is activated, the activation peptide is released causing an increase in the mobility of the dynamic flap (Fig. 1.6). The mobility of this region ultimately results in an irreversible conformational change that inactivates TAFIa to form inactivated TAFI (TAFIai).

In addition to Arg92, other thrombin and plasmin cleavage sites have been identified (Fig. 1.5). The additional thrombin cleavage site is Arg302, while Arg302, Lys327 and Arg330 have been identified as plasmin cleavage sites [214-216]. These sites were originally thought to be responsible for the inactivation of TAFIa. Mutation of the thrombin cleavage site (Arg302Gln) decreased the stability of TAFIa [214]. Therefore, this indicated that cleavage at Arg302 was not responsible for the inactivation of TAFIa. Furthermore, additional plasmin cleavage sites did not prevent inactivation [214-216]. These results confirmed that TAFIa inactivation was not a result of enzymatic cleavage by thrombin or plasmin [216]. Instead, it is thought that the conformation change exposes cryptic cleavage sites (*viz.* Arg302) that make TAFIai more susceptible to proteolytic cleavage.

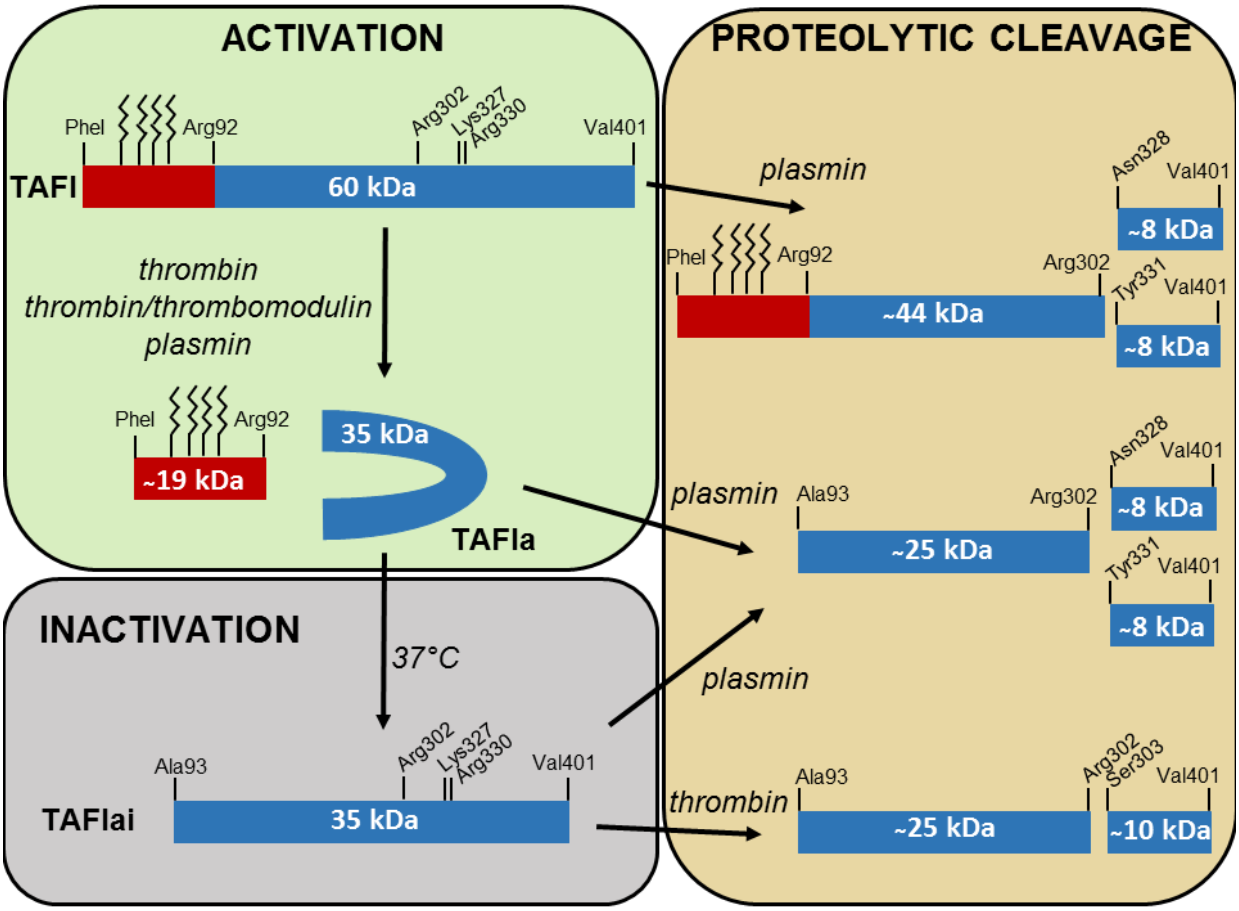

Figure 1.5: Activation, inactivation and proteolytic cleavage of TAFI.
The activation peptide of TAFI is shown in red and the catalytic domain is shown in blue. Four *N*-linked glycosylation sites in the activation peptide are depicted. TAFI is activated to TAFIa by proteolytic cleavage at Arg92 by thrombin, plasmin or thrombin/TM. Inactivation of TAFIa resulting in TAFIai is caused by an irreversible conformational change associated with loss of enzymatic activity. TAFI, TAFIa and TAFIai are also susceptible to proteolytic cleavage at sites distinct from Arg92. In addition to Arg92, thrombin is able to cleave TAFIai at Arg302. Plasmin cleavages sites in TAFI(a) are Arg302, Lys327 and Arg330. Adapted from reference [217].

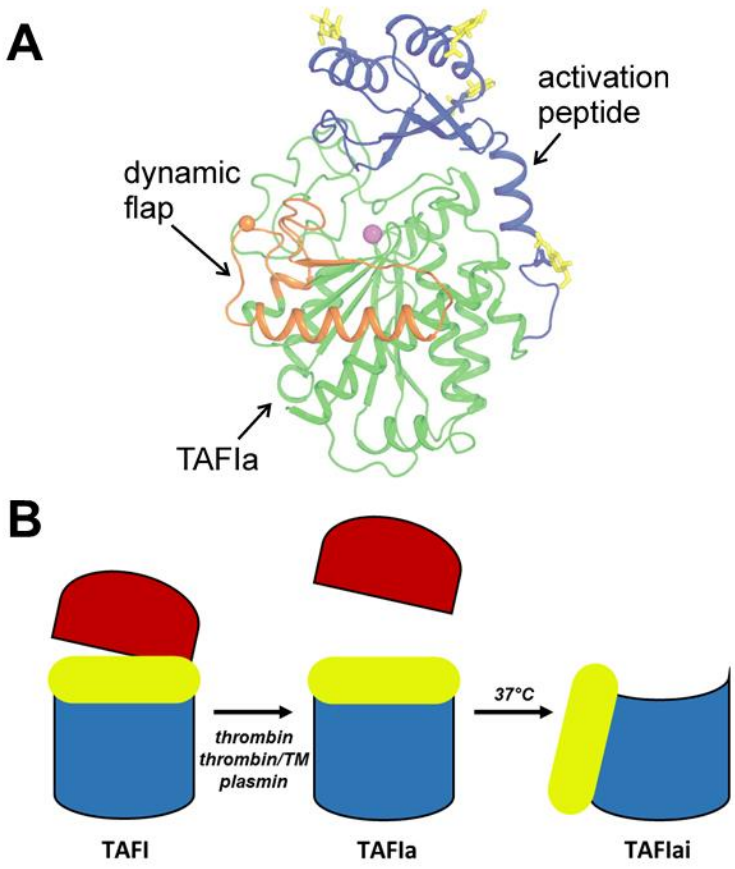

Figure 1.6: Structure of TAFI and inactivation of TAFIa.
A) TAFI illustrated as a ribbon structure. The activation peptide is shown in blue and 4 N-linked glycans are illustrated in yellow. When activated to TAFIa (shown in green), the activation peptide is cleaved. The dynamic flap region (amino acids 296-350) is shown in orange and the catalytic zinc ion is illustrated as a pink sphere. B) TAFI consists of the activation peptide (in red), the catalytic domain (in blue) and the dynamic flap region (in yellow). Activation peptide of TAFI is cleaved by thrombin, plasmin or thrombin/TM complex to form TAFIa. The dynamic flap region is composed of amino acids 296-350, which are stabilized by residues within the activation peptide of TAFI. When the activation peptide is released this region possess high mobility at 37°C, which results in an irreversible conformational changes that inactivates the enzyme, forming TAFIai. Adapted from reference [200].

As previously mentioned, two SNPs within the coding region of *CPB2* correspond to amino acid substitutions. The polymorphism resulting in Thr325Ile causes a change in the stability of TAFIa; note that residue 325 is within the dynamic flap. While Thr325 possesses a half-life of 8 minutes at 37°C, Ile325 nearly doubles the half-life of TAFIa to 15 minutes [168, 218]. The anti-fibrinolytic potency of the Ile325 variant is correspondingly greater. Studies have demonstrated that this polymorphism allows for more favourable interaction between the dynamic flap and the catalytic domain [213]. Intriguingly, individuals with the more stable variant of TAFIa have lower plasma levels of TAFI [168, 219], perhaps because of linkage disequilibrium between the SNP encoding the Thr325Ile substitution and other SNPs linked to differences in *CPB2* expression.

Several studies have used site-directed mutagenesis to identified key amino acids within the dynamic flap involved in the stability of TAFIa. Mutagenesis of Ser305 to Cys and Thr329 to Ile both result in an increase in intrinsic stability of TAFIa. The stabilizing effect of Cys305 is attributed to a more favourable interaction with aromatic residues in proximity to amino acid 305 [220]. Molecular modeling demonstrated that while Thr329 is exposed, Ile at this position is buried within the structure, allowing for favourable hydrophobic interactions with the catalytic domain. When these stabilizing mutations were combined with the naturally occurring stable isoform (Thr325Ile), the resulting intrinsic instability increased the half-life to 70 minutes at 37°C [220]. In addition, Knecht *et al.* observed that amino acid substitutions His333Tyr and His335Gln together increased the half-life of TAFIa to 90 minutes at 37°C [221]. When all five stabilizing mutations were combined (Ser305Cys, Thr325Ile, Thr329Ile His333Tyr and His335Gln – into a variant known as CIIYQ), the intrinsic instability markedly increased, corresponding to a half-life

of 1140 minutes at 37°C [222]. Recently, these mutations were combined with deletion of amino acids 1-73, which further increased the half-life to approximately 1980 minutes at 37°C [209]. This stable deletion mutant of TAFI illustrates the importance of amino acids Ala74-Arg92 of the activation peptide in stabilization of the catalytic domain, as exposing this variant to thrombin significantly decreased the stability [209].

To date, there are no known physiological inhibitors of TAFIa. The intrinsic instability of TAFIa resulting from its spontaneous conformational change is thought to the be primary regulator of TAFIa activity. Given that TAFIa is a basic carboxypeptidase, lysine and arginine analogs act as competitive inhibitors of TAFIa. Specifically, the arginine analogs 2-mercaptomethyl-3-guanindinoethylthiopropanoic acid (MERGEPTA) and 2-guanindinoethylmercaptosuccinic acid (GEMSA) and the lysine analog ε-aminocaproic acid (ε-ACA) are effective in inhibiting TAFIa [212, 223]. Unfortunately, these inhibitors are not specific, inhibiting other basic carboxypeptidases, including carboxypeptidase N (which, as previously mentioned, is also found in plasma) [224]. In addition to these analogs, natural carboxypeptidase inhibitors in potato, leech and tick have been identified [225-227].

Potato tuber carboxypeptidase inhibitor (PTCI) is a 39-amino acid peptide which has a molecular mass of 4.5 kDa [225]. It is thought that this potato peptide is important in inhibiting both endogenous and foreign carboxypeptidases. PTCI is a competitive inhibitor of TAFIa, as well as pancreatic carboxypeptidases A and B [228]. In the context of plasma, PTCI is specific to TAFIa, as it does not target carboxypeptidase N. PTCI has a 27 amino acid globular core with a protruding 5-amino acid carboxyl terminal tail, which is important for its inhibitory effects [229, 230]. The inhibitory mechanism of PTCI has been studied in

the context of carboxypeptidase A. Based on X-ray crystallography, the Val-38-Gly-39 bond of PTCI is hydrolyzed and the inhibitor remains trapped in the active site of carboxypeptidase A [229]. The new carboxyl-terminal Val-38 interacts with the zinc in the active site. Although similar studies have not been done with TAFIa, it is expected that the inhibitory mechanism is similar, given that the K_i values for TAFIa and carboxypeptidase A are both within the same nanomolar range [212].

Importantly, the above mentioned competitive inhibitors of TAFIa are problematic in that in addition to inhibiting the activity of the enzyme, they have all been shown to increase the stability [212]. Accordingly, these reversible inhibitors also protect TAFIa from proteolytic cleavage [214]. Studies with these inhibitors have demonstrated that they have a biphasic effect [231]. This biphasic effect is dependent on the concentration of TAFI and the inhibitor. At low concentrations these inhibitors enhance the effect of TAFIa, by increasing the half-life of the enzyme. At high concentrations these inhibitors are able to effectively inhibit the activity of TAFIa, although the half-life is increased [232]. In addition to the inhibitors, glycosaminoglycans, such as heparin, have also been shown to increase the half-life of TAFIa [202]. Based on crystal structure analysis, five sulfate ions were found bound to the dynamic flap, indicating that stabilization of TAFIa by heparin may result from direct binding to the dynamic flap region [233].

1.5.5 Biological Functions of TAFIa

TAFIa is a basic carboxypeptidase, cleaving carboxyl-terminal lysine and arginine residues from protein and peptide substrates, including fibrin degradation products (FDPs), plasminogen receptors, bradykinin, anaphylatoxins, thrombin-cleaved osteopontin and plasmin-cleaved chemerin [69, 129, 234-236]. TAFIa cleaves carboxyl-terminal lysines

from partially degraded fibrin, residues that mediate positive feedback in the fibrinolytic cascade by (i) accelerating tPA-mediated plasminogen activation; (ii) promoting plasmin-mediated Glu- to Lys-plasminogen conversion; and (iii) protecting plasmin from inhibition by antiplasmin [129, 237-239]. Accordingly, TAFIa attenuates fibrinolysis and hence promotes clot stability. Importantly, TAFIa attenuates fibrinolysis through a threshold-dependent mechanism [240]. As long as the concentration of TAFIa remains at or above the threshold, fibrinolysis is successfully attenuated. If the concentration of TAFIa falls below the threshold concentration, the number of carboxyl-terminal lysine residues on fibrin increases, accelerating fibrinolysis. Inhibition of fibrinolysis explains why elevated concentrations of TAFI have been associated with thrombotic disorders (see above). However, the antifibrinolytic activity of TAFIa may also be necessary for normal hemostasis. It has been demonstrated that in hemophilia, premature lysis of clots occurs because not enough thrombin is produced to activate sufficient TAFI to stabilize the clot [241-243].

TAFI deficient mice have been used to elucidate potential roles for TAFI *in vivo*. TAFI knockout mice do not show any overt abnormalities, developing and reproducing like their wild-type counterparts [244]. These mice however do show phenotypic differences when challenged. Several studies were completed to assess the effect of TAFI on models of fibrinolysis. Specifically, increased fibrinolysis was observed in TAFI knockout mice using a batroxobin-induced pulmonary embolism model [245]. Furthermore, a thromboembolism model demonstrated increased fibrinolysis in mice with a TAFI deficient background and double knockout of TAFI and PAI-1 [246]. This study

attributed the pro-fibrinolytic phenotype predominantly to the TAFI deficiency in mouce backgrounds.

Along with its role in balancing coagulation and fibrinolysis, TAFI also plays a role in down regulating inflammation. *In vitro*, TAFIa cleaves arginine residues from bradykinin and anaphylatoxins C3a and C5a, which results in inactivation of these inflammatory mediators [223, 234]. Studies in mice have also shown that they may also be substrates of TAFIa *in vivo* [247, 248]. Anaphylatoxins play a role in neutrophil recruitment and activation during inflammation [249]. Using an *E. coli*-induced sepsis model, TAFI deficiency resulted in enhanced neutrophil activity, correlating with elevated levels of C5a [250]. Additionally, increased levels of C5a were also observed in TAFI knockout mice with LPS-induced acute lung injury [251]. These results demonstrate a potential role for TAFI in inflammation.

TAFI deficiency has also been shown to affect wound healing *in vivo*. Using a cutaneous wound healing assay, te Velde and coworkers observed a delay in wound healing in mice with TAFI deficiency [252]. Keratinocyte migration is an important mechanism in cutaneous healing, which was impaired in TAFI deficient mice. This result may be attributed to enhanced fibrin clearance due to lack of TAFI, removing the matrix that provided a scaffold along which the cells migrated [253]. The study also examined the effect of TAFI deficiency in healing of colonic anastomoses [252]. This form of healing was also found to be impaired in TAFI deficient mice. Intriguingly, unlike cutaneous healing, this type of wound healing depends on mechanisms such as angiogenesis and ECM remodelling, which are mediated in part by the plasminogen activation system [252]. Importantly, TAFIa has been shown to modulate plasminogen activation *in vivo* [254].

Taken together, TAFI has several potential roles in mediating fibrinolysis, inflammation and wound healing. Regulation of fibrinolysis and wound healing are mediated by the ability of TAFI to attenuate plasminogen activation. Additional biological roles of TAFI may be identified through its ability to inhibit plasmin formation.

1.5.6 TAFI in Cancer and Angiogenesis

The role of TAFI in cancer has not been well-characterized. The majority of studies have examined plasma concentrations of TAFI and/or SNPs in patients with different types of cancer, with a specific focus on the contribution of TAFI to the thromboembolic complications that frequently accompany cancer. Hataji *et al.* demonstrated that plasma TAFI levels were increased in patients with lung cancer compared to healthy individuals [255]. The same study found that TAFI concentrations were higher in patients with small cell carcinoma compared to adenocarcinoma or squamous cell carcinoma. Intriguingly, they also found that plasma TAFI levels were higher in patients that responded to chemotherapy compared to non-responders. Unfortunately, this study did not examine TAFI levels in the context of metastasis. Another study also found an increase in plasma TAFI levels in patients with non-small cell lung cancer [256]. This study did not find any correlations with TAFI levels and stage of the disease. Eser *et al.* found no difference in TAFI concentrations in patients with gastric cancer compared to healthy controls [257]. However, an independent study by Fidan *et al.* found that TAFI levels were higher in gastric cancer patients when compared to healthy individuals [258]. Balcik *et al.* examined serum levels of TAFI in patients diagnosed with multiple myeloma and found an increase in patients compared to healthy controls [259]. It is speculated that increased plasma TAFI

concentrations may contribute to the fibrinolytic deficiency in cancer patients. However, increased levels may not be the cause but instead a consequence of disease progression, as there are many factors affecting *CPB2* gene expression and regulation that would ultimately influence TAFI protein levels. Meijers *et al.* showed that plasma concentrations of TAFI in patients with acute promyelocytic leukemia were similar to control groups [260]. However, they also determined that while TAFI levels were the same, there was a significant reduction in TAFI activity in patient samples.

TAFI levels in breast cancer patients have been examined in several studies. TAFI levels were found to be increased in breast cancer patients compared to healthy controls [261]. Unfortunately, this study did not provide information related to disease stage or metastasis. A study by Chengwei *et al.* also found an increase in TAFI levels in patients with breast cancer [262]. Conversely, Kotschy *et al.* examined plasma TAFI levels in patients with colorectal and breast cancers and found they were similar to control samples [263].

Associations with the Thr325Ile TAFI polymorphism have been examined in different cancer types. A large study of ovarian cancer found no significant association between the *CPB2* +1040C/T SNP and the incidence of ovarian cancer [264]. In contrast, the prevalence of the *CPB2* +1040C/T polymorphism was found to be less in patients with oral squamous cell carcinoma than in healthy controls [265]. It may be that the presence of the more stable isoform of TAFI may protect individuals from the onset of oral cancer. Conversely, two studies in breast cancer patients determined the *CPB2* +1040C/T polymorphism is positively associated with breast cancer [262, 266].

The lack of consistent results with respect to associations between TAFI levels or *CPB2* genotype and cancer is puzzling. It is possible that no such correlations exist and the few published studies are outliers. It is also possible that variations in study design, cancer type under investigation, and methods used to assay TAFI concentrations make it impossible to mine consensus from the literature. It is also notable that the existing studies are generally association studies with the occurrence of cancer. As such, they tell us nothing about whether differences in TAFI levels are the cause or the consequence of cancer incidence, or whether the differences are causally related or attributable to a chance association of TAFI levels with something else that is causally related. Finally, these studies do not provide any mechanistic insights, and do not address the key question of the role of TAFI in metastasis.

There have been few studies that focus on the biological functions of TAFI in cancer. A study in HT1080 fibrosarcoma cells found that TM attenuated invasion of these cells through promotion of TAFI activation [267]. On the other hand, no differences were seen in TAFI knockout mice compared to controls when observing tumour growth and metastasis [268]. However, this study was carried out using cell lines that do not support TAFI activation even in the presence of TAFI and therefore a role for TAFI cannot be ruled out. Intriguingly, mice with a double knockout background of TAFI and apolipoprotein-E (apoE) were found to develop hepatic tumours when treated with streptozotocin, a diabetogenic and carcinogenic agent [269]. Tumours were only observed in double knockout mice treated with steptozotocin and not in control mice or mice with the single knockout. Interestingly. apoE knockout mice treated with streptozotocin had increased plasma TAFI levels. Therefore, in the double knockout system, lack of TAFI may

contribute to tumourigenesis. This study also found that double knockout mice treated with streptozotocin has increased plasma concentrations of TGF-β1, which can promote cancer cell invasion and metastasis [270].

Several studies have also hinted at a potential role for TAFI in angiogenesis. TAFIa was found to decrease *in vitro* bFGF-induced endothelial cell tube formation on a plasma clot matrix, by inhibiting plasminogen activation [271]. This result implies that TAFI may inhibit tube formation in the tumour microenvironment. Furthermore, studies *in vivo* have demonstrated a role for TAFI in regulating plasmin-mediated migration of leukocytes and keratinocytes during would healing [252, 272]. TAFI may also regulate plasmin-mediated migration of endothelial cells during tumour angiogenesis.

1.6 Rationale and Objectives

TAFIa has a known biological function of attenuating plasminogen activation in the context of fibrinolysis and wound healing [129, 252]. Plasmin has a well-understood function in cancer progression, facilitating cell invasion and metastasis through degradation of the ECM and basement membrane [99]. Plasmin generation in the tumour microenvironment is enabled by plasminogen receptors, that accelerate activation of plasminogen to plasmin [62]. TAFIa has been shown to cleave carboxyl-terminal lysine residues on these receptors, inhibiting plasminogen activation [69]. The ability of TAFIa to attenuate plasminogen activation on the cell surface points to a potential role for TAFI in inhibiting the metastatic functions of plasmin, namely MMP and growth factor activation, ECM degradation and angiogenesis (Fig. 1.7). Furthermore, the endothelial cofactor TM is described as an anti-metastatic factor [142]. The anti-metastatic effects of

TM are attributed to its thrombin-binding domain, which is responsible for the activation of TAFI and protein C [144]. Studies have not determined which substrate of the thrombin/TM complex is responsible for the anti-metastatic effects of TM. We therefore hypothesize that (i) the anti-metastatic effects of TM result from its ability to activate TAFI and (ii) TAFIa functions as an anti-metastatic and anti-angiogenic factor through inhibition of plasminogen activation.

To address these hypotheses, the research objectives of this dissertation are:

1) To examine the effect of TAFIa on metastatic behaviours of breast cancer cells.
2) To assess the role of TAFIa on angiogenic potential of endothelial cells and endothelial-breast cancer cell co-cultures.
3) To determine the effect of thrombomodulin on metastatic and angiogenic behaviours in breast cancer cells.

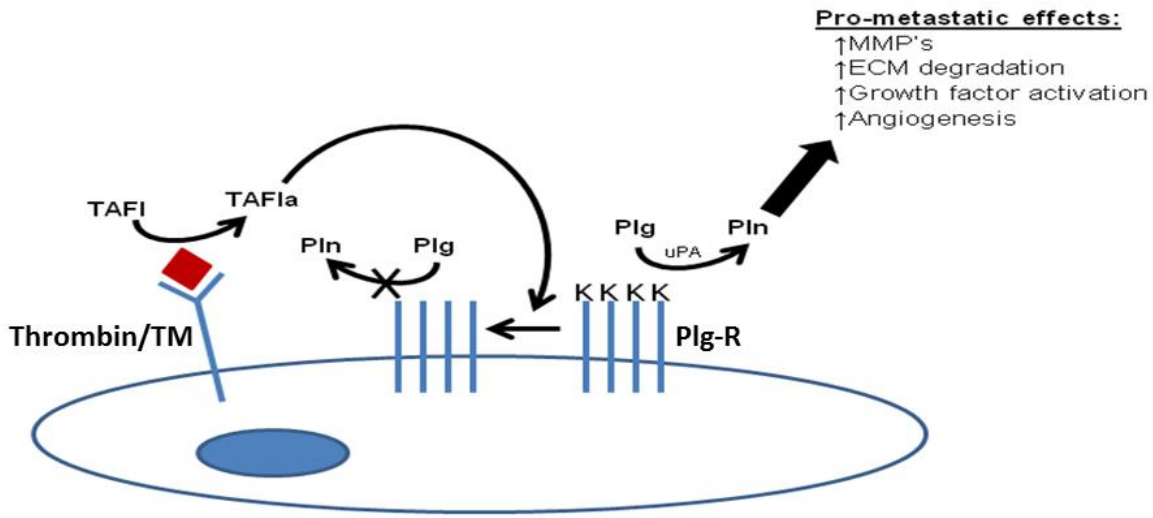

Figure 1.7: Hypothesis of TAFI in the tumour microenvironment.
TAFI is activated to TAFIa on the cancer cell surface by the thrombin/TM complex. Plasminogen (Plg) is activated to plasmin (Pln) by uPA when bound to plasminogen receptors (Plg-R) on the cell surface. Pln has pro-metastatic effects, including increased MMP secretion and activation, increased ECM degradation, increased growth factor activation and increased angiogenesis. TAFIa cleaves carboxyl-terminal lysine residues from Plg-R on the cell surface. This inhibits Plg conversion to Pln. This would therefore inhibit the pro-metastatic effects of Pln.

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Chapter 2

Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) attenuates breast cancer cell metastatic behaviors through inhibition of plasminogen activation and extracellular proteolysis

2.1 Summary

Background: Thrombin activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen, which can be converted to activated TAFI (TAFIa) through proteolytic cleavage by thrombin, plasmin, and most effectively thrombin in complex with the endothelial cofactor thrombomodulin (TM). TAFIa is a carboxypeptidase that cleaves carboxyl terminal lysine and arginine residues from protein and peptide substrates, including plasminogen-binding sites on cell surface receptors. Carboxyl terminal lysine residues play a pivotal role in enhancing cell surface plasminogen activation to plasmin. Plasmin has many critical functions including cleaving components of the extracellular matrix (ECM), which enhances invasion and migration of cancer cells. We therefore hypothesized that TAFIa could act to attenuate metastasis.

Methods: To assess the role of TAFIa in breast cancer metastasis, *in vitro* migration and invasion assays, live cell proteolysis and cell proliferation using MDA-MB-231 and SUM149 cells were carried out in the presence of a TAFIa inhibitor, recombinant TAFI variants, or soluble TM.

Results: Inhibition of TAFIa with potato tuber carboxypeptidase inhibitor increased cell invasion, migration and proteolysis of both cell lines, whereas addition of TM resulted in a decrease in all these parameters. A stable variant of TAFIa, TAFIa-CIIYQ, showed enhanced inhibitory effects on cell invasion, migration and proteolysis. Furthermore, pericellular plasminogen activation was significantly decreased on the surface of MDA-MB-231 and SUM149 cells following treatment with various concentrations of TAFIa.

Conclusions: Taken together, these results indicate a vital role for TAFIa in regulating pericellular plasminogen activation and ultimately ECM proteolysis in the breast cancer microenvironment. Enhancement of TAFI activation in this microenvironment may be a therapeutic strategy to inhibit invasion and prevent metastasis of breast cancer cells.

2.2 Background

Metastasis is the process by which malignant cells migrate from the site of the primary tumor to distant parts of the body [1]. This phenomenon is responsible for the majority of breast cancer related deaths. The breast tumor microenvironment facilitates metastasis by providing the necessary factors (such as stromal cells, signaling molecules, and proteolytic enzymes) to initiate the metastatic process. Importantly, metastasis is mediated by the interaction between the tumor cells, stromal cells and the extracellular matrix (ECM) [2, 3]. Proteins in the tumor microenvironment influence the progression of cancer by providing a favourable environment for ECM degradation. Specifically, the presence of proteases such as plasmin and matrix metalloproteinases (MMPs) mediate extracellular proteolysis, resulting in ECM degradation [4, 5]. Degradation of the ECM promotes cell migration and invasion and also releases latent growth factors that stimulate cell proliferation [1].

The plasminogen activation system (PAS) plays a vital role in extracellular proteolysis. Plasminogen is a zymogen that is converted to its active form plasmin by urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) [4], with uPA playing the more important role in pericellular plasminogen activation [6]. Plasmin is a serine protease of broad specificity that cleaves multiple substrates, including ECM proteins, latent growth factors and a number of pro-MMPs [4]. Plasmin therefore is considered a pro-metastasis enzyme [7]. The PAS is active in many types of cancers, including breast cancer [4]. Specifically, uPA and its receptor, urokinase plasminogen activator receptor (uPAR) are expressed in tumor cells, as well as tumor-associated stromal

cells [4, 8]. Developing methods to target the PAS could be key in inhibiting cancer cell invasion and metastasis [9].

Thrombin activatable fibrinolysis inhibitor (TAFI), also known as carboxypeptidase B, U or R [10], is a plasma zymogen expressed mainly in the liver but also found in megakaryocytes (leading to a platelet pool of TAFI) and macrophages [11]. TAFI is converted to activated TAFI (TAFIa) by proteolytic cleavage at Arg92 either by thrombin, plasmin or thrombin in complex with the endothelial cofactor thrombomodulin (TM). TAFIa is a basic carboxypeptidase, which removes carboxyl-terminal arginine and lysine residues from peptide and protein substrates, including fibrin degradation products (FDPs), anaphylatoxins C3a and C5a, thrombin-cleaved osteopontin and cell-surface plasminogen receptors [12-15].

Intriguingly, TAFIa is intrinsically unstable, with a half-life of 8-15 minutes at 37°C. This instability is considered to be the physiological means of TAFIa inhibition, as no endogenous inhibitors exist [16]. Ceresa *et al.* characterized a mutant of TAFIa with a 180-fold enhancement in half-life compared to wildtype TAFIa [17]. This stable mutant of TAFIa contains five point mutations in the instability region, specifically S305C, T325I, T329I, H333Y, H335Q and is therefore named TAFI-CIIYQ.

The ability of TAFIa to cleave cell-surface plasminogen receptors raises the possibility that TAFIa could modulate the PAS in the tumor microenvironment, thereby attenuating the metastatic potential of the tumor cells. Furthermore, TM, the cofactor necessary for physiological activation of TAFI has been characterized as an anti-metastatic factor. *In vitro* studies have shown that TM can inhibit invasion of fibrosarcoma cells through activation of TAFI [18]. An important *in vivo* study demonstrated that the anti-

metastatic potential of TM could be attributed to its thrombin-binding domain [19]. In addition, expression of TM in breast cancer tumors is inversely correlated to malignancy [20]. Therefore, we hypothesized that the anti-metastatic potential of TM may be mediated by its ability to enhance the activation of TAFI. Here, we address this hypothesis using an *in vitro* model of the breast tumor microenvironment.

2.3 Methods

2.3.1 Cell Culture

MDA-MB-231 and HTB-126 cells (ATCC) were grown in Dulbecco's modified eagle medium (DMEM/F12) (Invitrogen), containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (10 units/mL penicillin G sodium, 10 µg/mL streptomycin sulfate and 25 ng/mL amphotericin B) (Invitrogen). SUM149 cells (a kind gift from Dr. Stephen Ethier, Karmanos Cancer Institute) were cultured in DMEM/F12 containing 5% FBS, 1% antibiotic-antimycotic, 10 µg/mL insulin (Sigma) and 0.5 µg/mL hydrocortisone (Sigma). MCF7 cells (ATCC) were grown in RPMI-1640 medium (Gibco) containing 10% FBS and 1% antibiotic-antimycotic. MCF10A (ATCC) and MCF10CA1a (obtained from the Karmanos Biobanking and Correlative Sciences Core) were maintained in DMEM-F12 media containing 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, 20 ng/ml human EGF and 5% (vol/vol) heat inactivated horse serum. Human embryonic kidney (HEK293) cells (ATCC) were cultured in minimum essential medium (MEM) (Gibco) supplemented with 5% FBS and 1% antibiotic-antimycotic. THP-1 acute monocytic leukemia cells (ATCC) were cultured in RPMI-1640 medium adjusted to contain 4.5 g/L glucose, 10 mM HEPES pH 7.4 and 1.0 mM sodium pyruvate, and supplemented with 10% (v/v) fetal bovine serum (ATCC), 1% (v/v) antibiotic-antimycotic, and 50 µM β-mercaptoethanol. THP-1 monocytes were differentiated by the addition of 100 nM phorbol 12-myristate 13-acetate to the medium for 72 hours. Human umbilical vein endothelial cells (HUVECs) (Lonza) were grown in EGM Complete Medium (Lonza). All cells were sub-cultured in 100 mm cell culture plates and were kept in an incubator maintaining conditions of 37°C, 5% CO_2.

2.3.2 Cloning, Expression and Purification of recombinant TAFI

The full-length TAFI-CIIYQ cDNA sequence [17] present in the pNUT vector [21] was used as a template for PCR amplification. To insert TAFI-CIIYQ in the pcDNA4A vector, unique *Pst*I and *Age*I restriction sites were engineered at the 5' and 3' ends of the sequence through PCR. PCR amplification was performed with Phusion HF DNA polymerase (New England Biolabs) as per manufacturer's protocol, using TAFI-CIIYQ-pNUT as the template (10 ng/µL) and the following primer pair: sense 5'-AAA CTG CAG TTG GGA TGA AGC TTT GC-3' and anti-sense 5'-GGA CCG GTA ACA TTC CTA ATG ACA TGC CAA G-3'. Using *Pst*I and *Age*I, TAFI-CIIYQ cDNA was ligated into the pcDNA4A plasmid in-frame with a carboxyl-terminal His_6-tag-encoding sequence. TAFI-WT [21] was cloned into pcDNA4A using the same method.

HEK293 cells were seeded into a 6-well plate at a density of 1.5 $x10^6$ cells per well. Cells were transfected using 1 µg of TAFI-pcDNA4A expression plasmids and Mega Tran 1.0 (OriGene) according to manufacturer's protocol. Stably-expressing cell lines were selected by culturing in the presence of 100 µg/mL zeocin (Invitrogen). Stable cell lines were grown in roller bottle culture for the production of recombinant TAFI in OptiMEM (Gibco) supplemented with 1% antibiotic/antimycotic.

Recombinant TAFI (rTAFI) was purified using a Ni^{2+} Sepharose column (GE Healthcare Life Sciences), utilizing the His_6-tag at the carboxyl-terminal of the protein. Conditioned medium harvested from the cells was supplemented with 50 mM NaH_2PO_4, 0.5 M NaCl, 1 mM β-ME 10% glycerol, pH 7.9 (buffer A), containing 5 mM imidazole. The column was washed and eluted with wash and elution buffers, which were buffer A

containing 10 mM and 400 mM imidazole, respectively. Eluted rTAFI was dialyzed overnight at 4ºC against HBS (0.02 M HEPES pH 7.4, 0.15 M NaCl) containing 5% glycerol. Dialyzed rTAFI was then concentrated by ultracentrifugation using Amicon Ultra Centrifugal Filters (Fisher Scientific) at 4ºC with a buffer exchange using HBS containing 0.01% (v/v) Tween-80 (HBST).

2.3.3 RNA Extraction and Quantitative Real-time PCR

RNA was extracted from various cell lines using RNeasy Plus Mini Kit (Qiagen), as per the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was completed using iTaq one-step RT-PCR kit with SYBR® green (BioRad). The following primers were used for qRT-PCR: TM sense: 5' CCC TGA ACA AGA ATT GGA AGC T 3'; TM antisense: 5' GGA GCC TAG GAT TCT GCA TTT CTA 3'; TAFI sense: 5' GCT GCC GGA GCG TTA CAT 3'; TAFI antisense: 5' CAT TCC TAA TGA CAT GCC AAG CT 3'; GAPDH sense: 5' GGA GCC AAA AGG GTC ATC ATC 3'; GAPDH antisense: 5' GTT CAC ACC CAT GAC GAA CAT G 3'.

2.3.4 Plasminogen Activation Assays

Plasminogen activation assays were performed as previously described by Romagnuolo *et al.* [22]. Briefly, MDA-MB-231 and SUM149 cells were grown to confluency in black, clear-bottom 96 well plates. Various TAFI concentrations were activated in Hank's buffer saline solution (HBSS) (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 1 g/L

glucose and 0.1% BSA) with 25 nM thrombin, 100 nM TM, and 5 mM $CaCl_2$ for 10 minutes at room temperature. Following the 10-minute incubation, thrombin was inhibited with PPAck. Cells were washed twice with HBSS. Activated TAFI (1-50 nM, final) was added to cells for 30 minutes at 37°C. Cells were then washed twice with HBSS and a solution containing 300 nM plasminogen (purified from human plasma by lysine-sepharose affinity chromatography as previously described [23]), 10 pM uPA (Calbiochem) and 40 mM of the fluorogenic plasmin substrate H-D-Val-Leu-Lys-AMC (Bachem). Hydrolysis of H-D-Val-Leu-Lys-AMC was monitored for an hour at 37°C at excitation and emission wavelengths of 370 nm and 470 nm, respectively.

2.3.5 Cell Invasion and Migration Assays

Cell culture inserts (BD Biosciences) with a pore size of 8 µm were coated with 2 mg/mL Cultrex reconstituted basement membrane extract (Trevigen) for 1 hour at room temperature. MDA-MB-231 and SUM149 cells (1×10^5) in serum-free medium were seeded into the upper chamber. Complete medium was added to the lower chamber with either 10 ug/mL potato tuber carboxypeptidase inhibitor (PTCI) (Sigma) or 10 nM rabbit TM (Haemotologic Technologies). Cells were allowed to invade for 24 hours at 37°C. Following 24 hours, non-invaded cells in the upper chamber were removed. Invaded cells were fixed with cold methanol and stained with 0.25% crystal violet. Images were taken of five fields of view with a 20× objective and cell numbers in each field were determined by counting.

For TAFIa treatment, recombinant TAFI was activated with 25 nM thrombin (Haematologic Technologies), 100 nM TM, and 5 mM $CaCl_2$ for 10 minutes at room

temperature. Thrombin was inhibited with 200 nM H-D-Phe-Pro-Arg chloromethylketone (PPAck; Calbiochem) and placed on wet ice. The activation mixture was supplemented with DMEM containing 10% FBS and 1% antibiotic and added to the lower chamber to achieve final TAFIa concentrations of 1, 5, 25, or 50 nM. Mock activations of rTAFI were carried out in the same manner but without thrombin.

Cell migration assays were performed using the same protocol as cell invasion assays, except that the cell culture inserts were not coated with Cultrex and the cells were seeded in the upper chamber at a lower density (3×10^4 cells/well).

2.3.6 Cell Metabolism Assay

MDA-MB-231 and SUM149 cells (2.5×10^3/well) were seeded into a 96 well plate. Cells were grown for 24 hours. PTCI or TM was then added to the cells in fresh medium for 24 hours. Following the 24 hours, WST-1 reagent (Roche) was added and incubated for 2 hours at 37°C. The absorbance was then measured at 450 nm using a plate-reading spectrophotometer.

2.3.7 Live-cell proteolysis assay

Live-cell proteolysis experiments were carried out as described by Victor *et al.* [24]. Round glass-bottom dishes (MatTek) were coated with Cultrex containing 25 µg/mL dye quenched (DQ)-collagen IV (Invitrogen). MDA-MB-231 cells and SUM149 cells were labeled with CellTracker Orange (Invitrogen) in serum-free medium for 1 hour. Cells were allowed to recover in complete medium before 2×10^4 (SUM149) or 1×10^4 cells (MDA-

MB-231) were seeded onto the Cultrex-coated glass. Dishes were then incubated at 37°C for 45 minutes. Following the incubation, complete growth medium containing 2% Cultrex was added to the cells, with or without 10 µg/mL PTCI. Cells were incubated at 37°C and 5% CO_2 for 48 hours. Following incubation, cells were imaged using an Olympus IX81 confocal microscope.

For TAFIa treatment, activated (50 nM) and mock-activated (50 nM) rTAFI mixtures were prepared as described above. Glass-bottom dishes were prepared and cells were seeded as described above. Each plate was supplemented with 2 mL of DMEM medium containing 10% FBS, 1% antibiotic, 2% Cultrex and the activation mixture with or without activated TAFI. Cells were incubated at 37°C and 5% CO_2 for 48 hours.

Quantitative analysis of the confocal microscopy data was performed using the following protocol. Each of the images (15- 20 depending on the size of the spheroid) of the z-stack in the experiment was initially in TIFF RGB format, which were then converted to 8-bit grayscale prior to processing. The images were then filtered to remove granulation using a median filter. The brightness curves were trimmed so that areas of high intensity did not discard other areas of interest when thresholding; the identical trimming method was conducted for each image. Each image was then filtered and tuned (again using the same parameters in each case), a threshold based on Otsu's technique [25] was applied to the image which converts it to a binary format. The resultant image contains an accurate depiction of the shape and contour of the areas of interest. At this stage, the areas of each image were then calculated by matrix summation. The average intensity of an image was then determined by applying the binary image as a mask to the original grayscale image and calculating the mean pixel intensity of the resulting matrix. Each pixel is represented

as an 8-bit grayscale value and therefore represented as a number between 0 and 255. This algorithm was applied to each image in the same exact manner without any modifications. The data were represented as a ratio of the intensity of the green fluorescence to the area of the cells, averaged over the z-stack.

2.3.8 Statistical Analysis

Data from the experiments were presented as means of at least 3 independent experiments ± standard error of the mean (SEM). Analysis of the data to identify statistically significant differences amongst experimental conditions was completed using one-way ANOVA with SPSS software (version 22); pairwise comparisons between conditions were performed using a Tukey post-hoc analysis. p values < 0.05 were considered statistically significant.

2.4 Results

2.4.1 TAFI and TM are expressed in breast cancer cell lines

Expression of *CPB2* (the gene encoding TAFI) was assessed in various breast cancer cell lines, using qRT-PCR (Figure 1). *CPB2* mRNA was detectable in all of the examined breast cancer cell lines, albeit at a lower level in all cases compared to the positive control THP-1 macrophages (Figure 1), which is correspondingly much lower than reported in liver or a cultured hepatoma cell line [11]. *CPB2* mRNA levels in the highly malignant and invasive MDA-MB-231, HTB-126, and MCF10ACA1a cell lines were comparable to mRNA levels in the non-invasive [26] MCF7 cell line. Therefore, levels of *CPB2* mRNA do not appear to show any relationship to the malignancy of the breast cancer cell lines.

It has been reported that TM expression is inversed correlated with malignancy in prostate, breast and lung cancer [27-29]. In contrast to *CPB2*, the expression levels of *THBD* (the gene encoding TM) were found to be generally inversely correlated to malignancy (Figure 1). This relationship is revealed when the cell lines are arranged in decreasing order of *THBD* expression from left to right, as the more malignant cell lines are on the right.

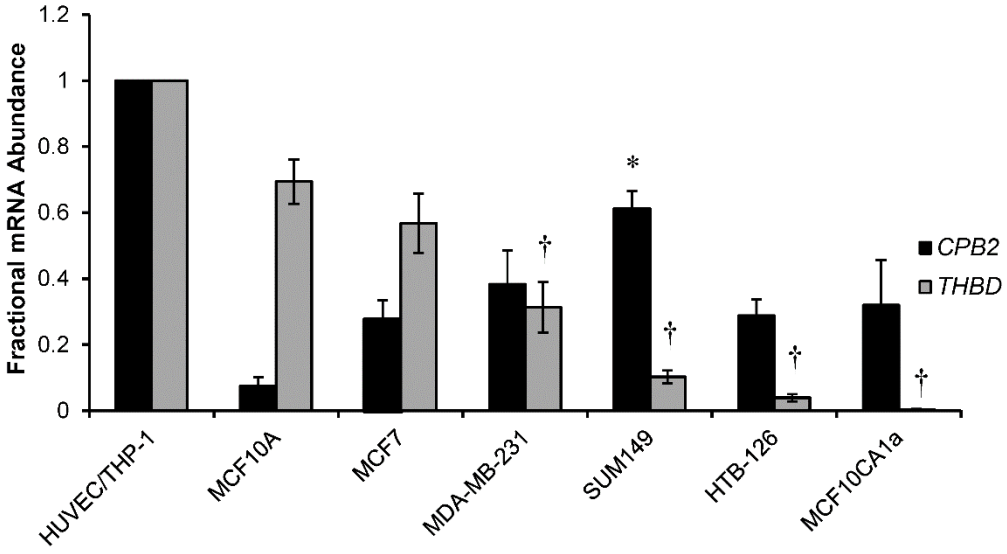

Figure 2.1: Expression of *CPB2* (TAFI) and *THBD* (thrombomodulin) mRNA in breast cancer cell lines.

RNA was extracted from various breast cancer cell lines and expression of *CPB2* and *THBD* was analyzed using qRT-PCR. Expression of *CPB2* and *THBD* were normalized to *GAPDH* mRNA levels in all cells. The data are expressed relative to THP-1 macrophages (*CPB2*) or HUVECs (*THBD*) and are the means ± SEM of 3-4 independent experiments, and are arranged in decreasing order of *THBD* expression from left to right. *: $p < 0.05$ versus MCF10A (*CPB2*) and †: $p < 0.05$ versus MCF10A (*THBD*).

2.4.2 TAFIa inhibits plasminogen activation on both MDA-MB-231 and SUM149 cell lines

Addition of TAFIa resulted in a decrease in plasminogen activation of up to 30% in both MDA-MB-231 and SUM149 cells (Figure 2). This decrease, however, was not strictly dose-dependent, as the magnitude of the effect tended to decrease at the highest concentrations of TAFIa. The ability of TAFIa to decrease cell surface plasminogen activation is consistent with its ability to decrease extracellular collagen proteolysis.

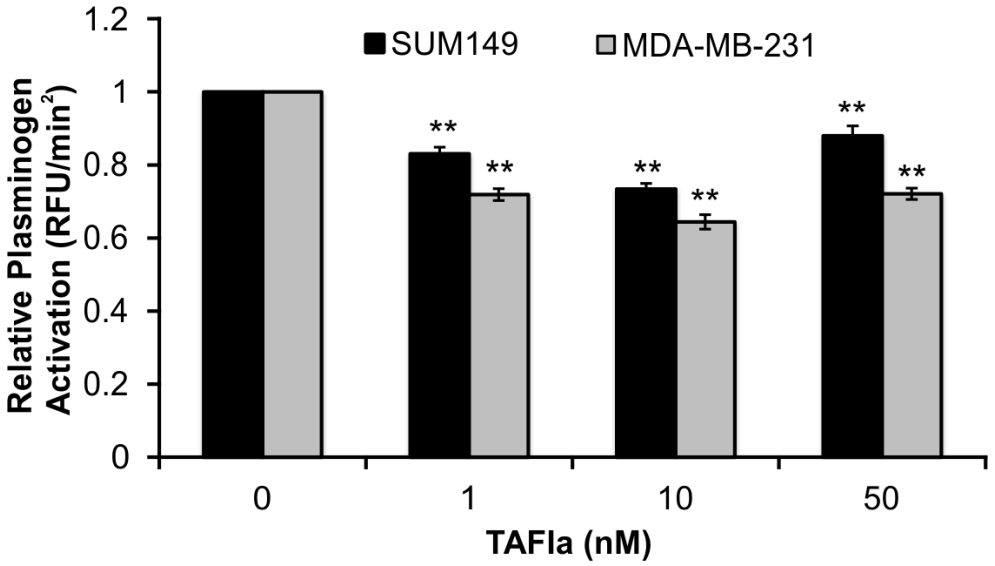

Figure 2.2: TAFIa inhibits pericellular plasminogen activation on breast cancer cell lines.

SUM149 (black bars) and MDA-MB-231 cells (grey bars) were treated with varying concentrations of TAFIa (1-50 nM) for 30 minutes at 37°C. Plasminogen activation assay was carried out with 300 nM plasminogen, 10 pM uPA and a fluorescent plasmin substrate. The rate of formation of plasmin (taken as the slope of the plot of change in fluorescence versus time squared) was monitored for 1 hour at 37°C. The data represent the means ± SEM of 4-5 independent experiments and are expressed relative to the control in the absence of TAFIa. **: $p < 0.01$ relative to control.

2.4.3 TAFIa directly inhibits cell invasion and migration of MDA-MB-231 and SUM149 cell lines

We examined the effect of TAFIa on cell invasion of MDA-MB-231 and SUM149 cell lines by inhibition of TAFIa using the specific competitive inhibitor PTCI. Both cell lines should be sensitive to the effects of PTCI as they both express TM and therefore presumably have the capacity to support TAFI activation. Inhibition of TAFIa using 10 μg/mL PTCI resulted in a significant increase in invasion (Figure 3A,B) and migration (Figure 4A,B) of both MDA-MB-231 and SUM149 cells. In addition, treatment with 10 nM of the cofactor TM resulted in an approximately 30% decrease in invasion in both SUM149 and MDA-MB-231 cell lines (Figure 3A,B) as well as decreases in migration of MDA-MB-231 and SUM149 cells by 30% and 20%, respectively (Figure 4A,B). These results indicate that modulation of TAFI activity can affect invasion and migration potential of breast cancer cells.

To directly assess the effect of TAFIa on cell invasion we used wild-type TAFI (TAFI-WT) as well as a stable variant (TAFI-CIIYQ) (Figure 3C,D). While treatment with activated TAFI-WT (TAFIa-WT) did not affect cell invasion, we observed a dose-dependent decrease in MDA-MB-231 cell invasion upon treatment with TAFIa-CIIYQ. The highest dose of TAFIa-CIIYQ (50 nM) resulted in an approximately 60% decrease in invasion of MDA-MB-231 cells (Figure 3C,D). Notably, this effect was only observed with the TAFIa enzyme and not the (non-activated) TAFI zymogen. In addition, we assessed the effect of TAFI and TAFIa on cell migration. We observed a dose-dependent decrease in cell migration of MDA-MB-231 cells when treated with either TAFIa-WT or TAFIa-CIIYQ (Figure 4C,D), although significance was only reached at 50 nM TAFIa-CIIYQ.

Treatment with the non-activated TAFI did not have an effect on migration, as we observed with cell invasion. These results demonstrate directly that TAFIa can inhibit breast cancer cell invasion and migration *in vitro*.

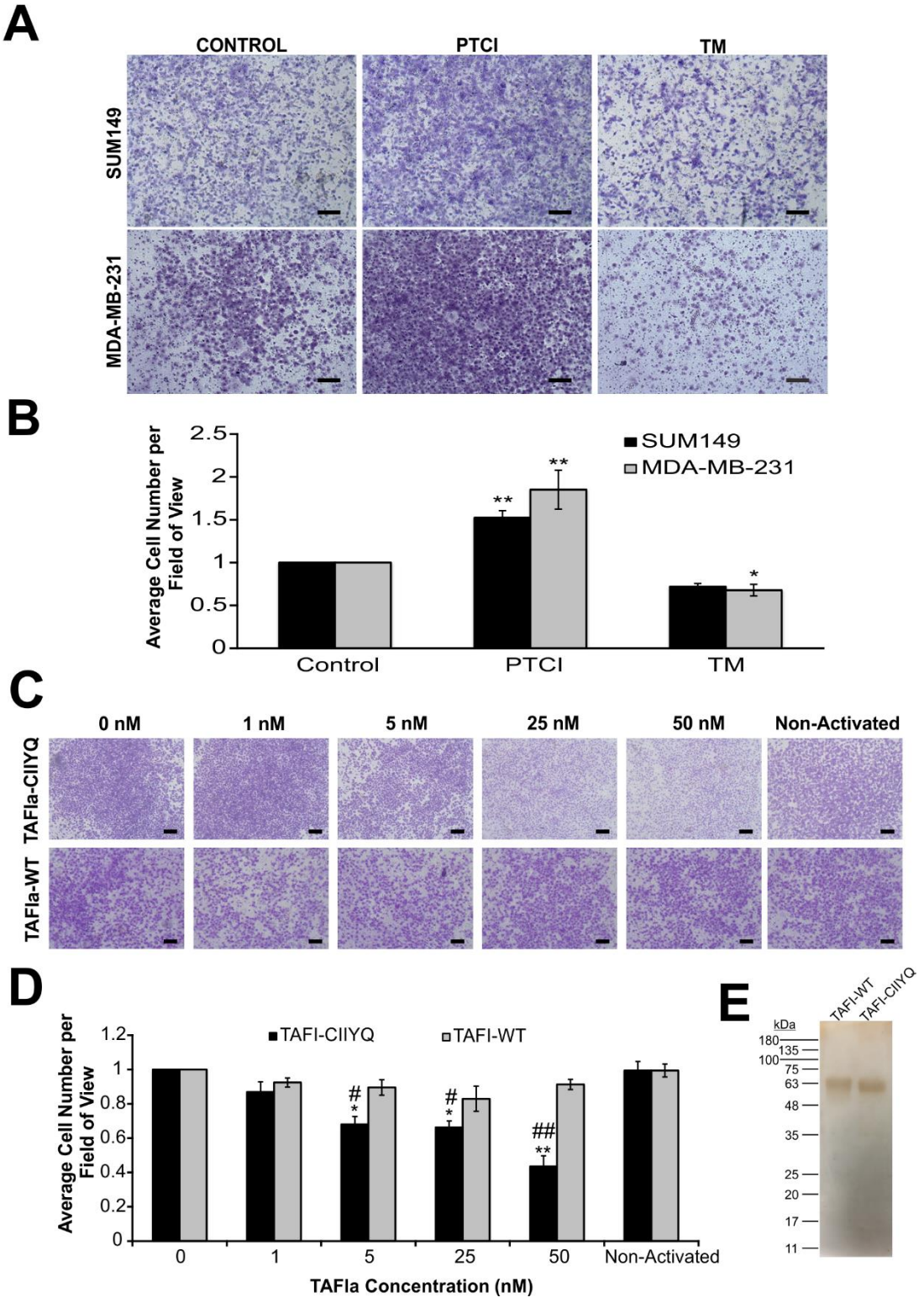
A
CONTROL
PTCI
TM
SUM149
MDA-MB-231
B
Average Cell Number per Field of View
SUM149
MDA-MB-231
Control
PTCI
TM
C
0 nM
1 nM
5 nM
25 nM
50 nM
Non-Activated
TAFIa-CIIYQ
TAFIa-WT
D
Average Cell Number per Field of View
TAFI-CIIYQ
TAFI-WT
TAFIa Concentration (nM)
E
TAFI-WT
TAFI-CIIYQ
kDa
180
135
100
75
63
48
35
25
20
17
11

Figure 2.3: The effects of PTCI, TM and TAFIa on breast cancer cell invasion.

(A) SUM149 and MDA-MB-231 cells were subjected to invasion assays in the presence of 10 µg/mL PTCI or 10 nM TM or in the absence of these additions (Control). The number of invaded cells were imaged and counted in five fields of view. Images shown were taken at 4× magnification. Scale bars: 200 µm. (B) Quantification of invaded cells, relative to control (absence of treatment). The data represent the average cell number per field of view ± SEM from at least 4 independent experiments. *: $p < 0.05$ and **: $p < 0.01$ relative to control. (C) Cells were treated with varying concentrations (1-50 nM) of either TAFIa-WT or TAFIa-CIIYQ or 50 nM mock-activated TAFI-WT or TAFI-CIIYQ (Non-Activated) for 24 hours. Invasion was imaged as in (A). (D) Quantification of invaded cells, relative to control (absence of TAFIa). The data represent the average cell number per field of view ± SEM from at least 3 independent experiments. *: $p < 0.05$ and **: $p < 0.01$ relative to control; #: $p < 0.05$ and ## $p < 0.01$ relative to TAFIa-WT. (E) To demonstrate the purity of the recombinant TAFI proteins, TAFI-WT and TAFI-CIIYQ (500 ng each) was subjected to SDS-PAGE on a 12% polyacrylamide gel followed by staining with silver. The positions of migration of molecular weight markers are shown to the left of the gel.

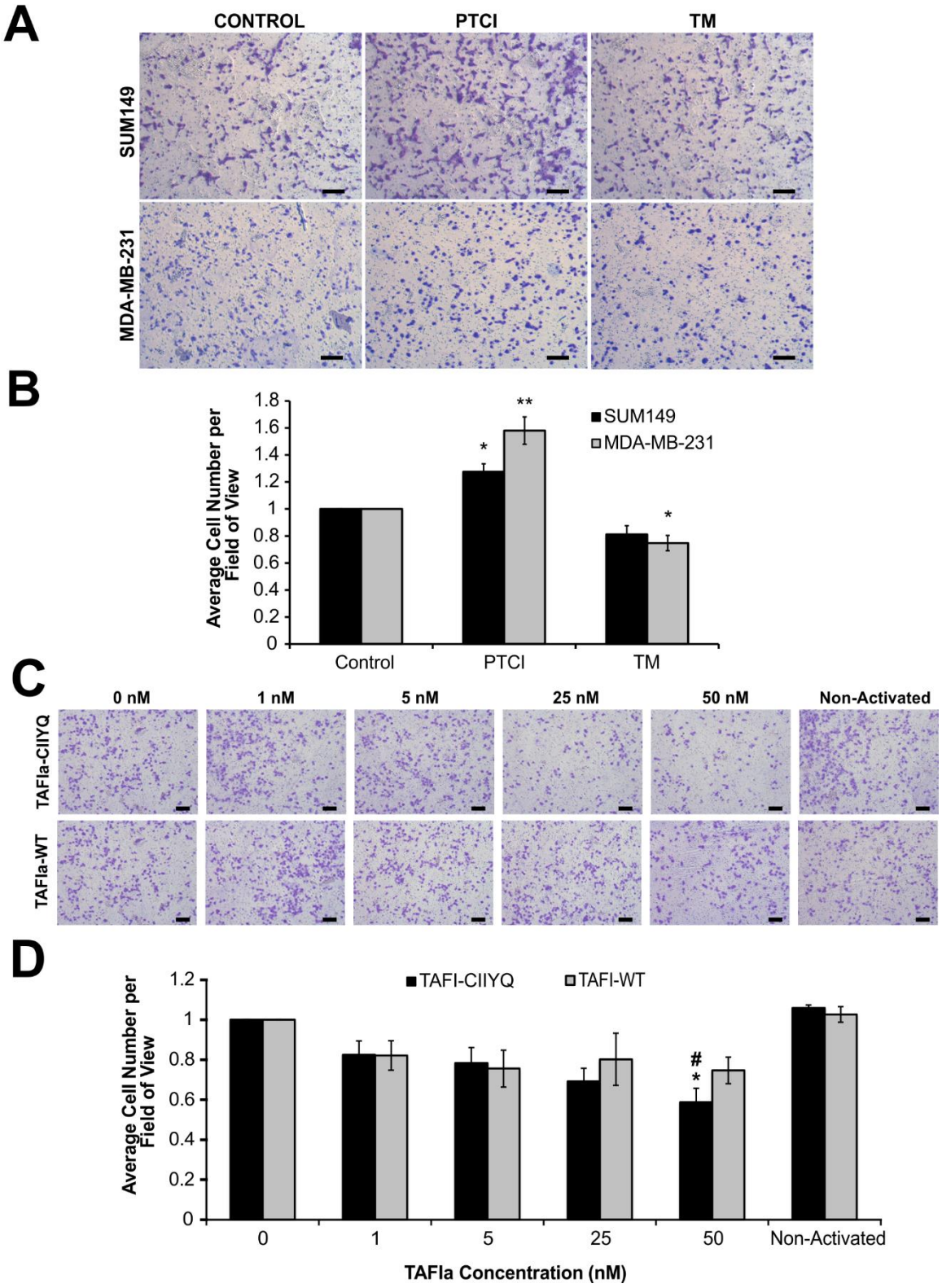
A
CONTROL
PTCI
TM
SUM149
MDA-MB-231
B
Average Cell Number per Field of View
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SUM149
MDA-MB-231
*
**
*
Control
PTCI
TM
C
0 nM
1 nM
5 nM
25 nM
50 nM
Non-Activated
TAFIa-CIIYQ
TAFIa-WT
D
Average Cell Number per Field of View
1.2
1
0.8
0.6
0.4
0.2
0
TAFI-CIIYQ
TAFI-WT
#
*
0
1
5
25
50
Non-Activated
TAFIa Concentration (nM)

Figure 2.4: The effects of PTCI, TM and TAFIa on breast cancer cell migration.
(A) SUM149 and MDA-MB-231 cells were subjected to migration assays in the presence of 10 µg/mL PTCI or 10 nM TM or in the absence of these additions (Control). The number of migrated cells were imaged and counted in five fields of view. Images shown were taken at 4× magnification. Scale bars: 200 µm. (B) Quantification of migrated cells, relative to control (absence of treatment). The data represent the average cell number per field of view ± SEM from at least 4 independent experiments. *: $p < 0.05$ and **: $p < 0.01$ relative to control. (C) Cells were treated with varying concentrations of either TAFIa-WT or TAFIa-CIIYQ (1-50 nM) for 24 hours. Cells were also treated with 50 nM mock-activated TAFI-WT or TAFI-CIIYQ (Non-Activated). Migration was imaged as in (A). (D) Quantification of migrated cells, relative to control in the absence of TAFIa. The data represent the average cell number per field of view ± SEM from at least 3 independent experiments. *: $p < 0.05$ relative to control; #: $p < 0.05$ relative to TAFIa-WT.

2.4.4 TAFIa does not affect breast cancer cell proliferation

We next wanted to determine whether manipulation of TAFI impacted cell proliferation. Treatment with 10 μg/mL PTCI had no effect while treatment with 10 nM TM slightly decreased cell proliferation (as measured by a metabolic assay), albeit to an extent that was not statistically significant (Figure 5). This result is in keeping with previous study conducted in melanoma cells [30]. As shown in Figure 5B, treatment of MDA-MB-231 cells with both variants of TAFI resulted in a slight, albeit again non-significant, decrease in cell proliferation. This result was independent of TAFI activation, as addition of TAFIa gave the same result as addition of the zymogen.

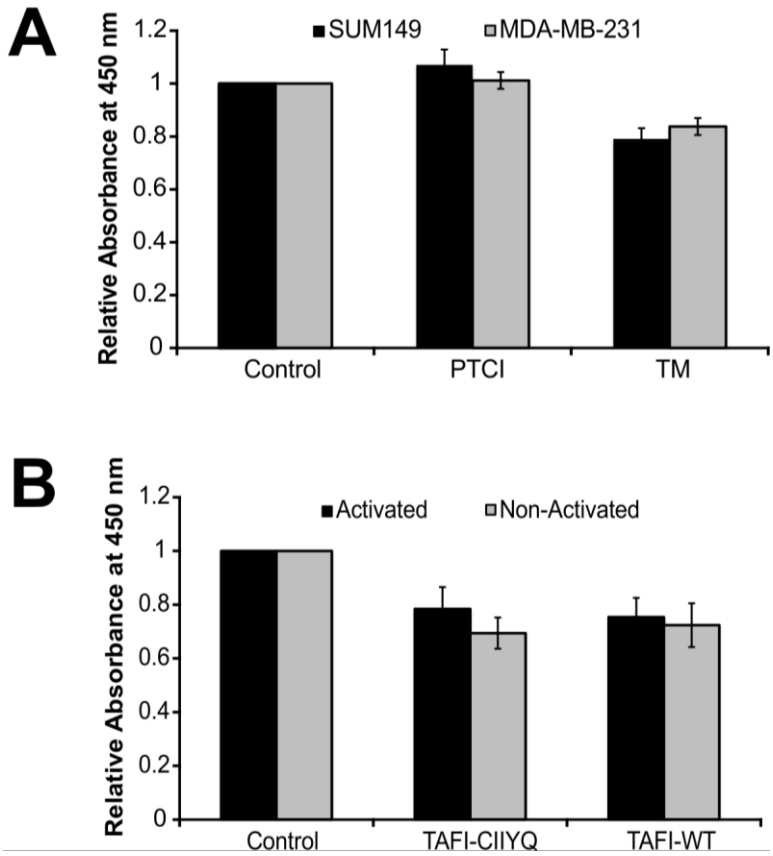

Figure 2.5: The effects of PTCI and TM on cell proliferation of breast cancer cell lines.

MDA-MB-231 and SUM149 cells were subjected to the WST-1 assay as an index of proliferation. (A) Cells were treated with 10 µg/mL PTCI or 10 nM TM, for 24 hours. (B) MDA-MB-231 cells were treated with 50 nM of the indicated TAFI variants, either with (Activated) or without (Non-Activated) prior treatment with thrombin-TM. Following the treatment, the WST-1 assay was conducted and metabolic activity of the cells assessed by measuring absorbance at 450 nm. The data represent the average cell number per field of view ± SEM from at least 3 independent experiments and are relative to the control in the absence of treatment.

2.4.5 TAFIa inhibits DQ-collagen type IV proteolysis by MDA-MB-231 and SUM149 cells

We examined the effect of TAFIa on degradation of DQ-collagen type IV by MDA-MB-231 and SUM149 cells. Inhibition of TAFIa using 10 μg/mL PTCI resulted in a significant increase in collagen IV proteolysis by both MDA-MB-231 and SUM149 cells (Figure 7). DQ-collagen degradation products were localized both intracellularly (represented by the yellow in the merged images) and extracellularly.

Next, we sought to directly investigate the effect of TAFIa on DQ-collagen type IV degradation. MDA-MB-231 cells were treated with either activated or mock-activated TAFI variants, TAFI-WT or TAFI-CIIYQ (Figure 8). Intriguingly, addition of TAFI-CIIYQ resulted in a decrease in collagen type IV proteolysis of approximately 30%, irrespective of whether this variant was activated or not (Figure 8B). Addition of wild-type TAFI or TAFIa had no significant effect on proteolysis. Taken together, these results demonstrate a role for TAFIa in preventing collagen degradation.

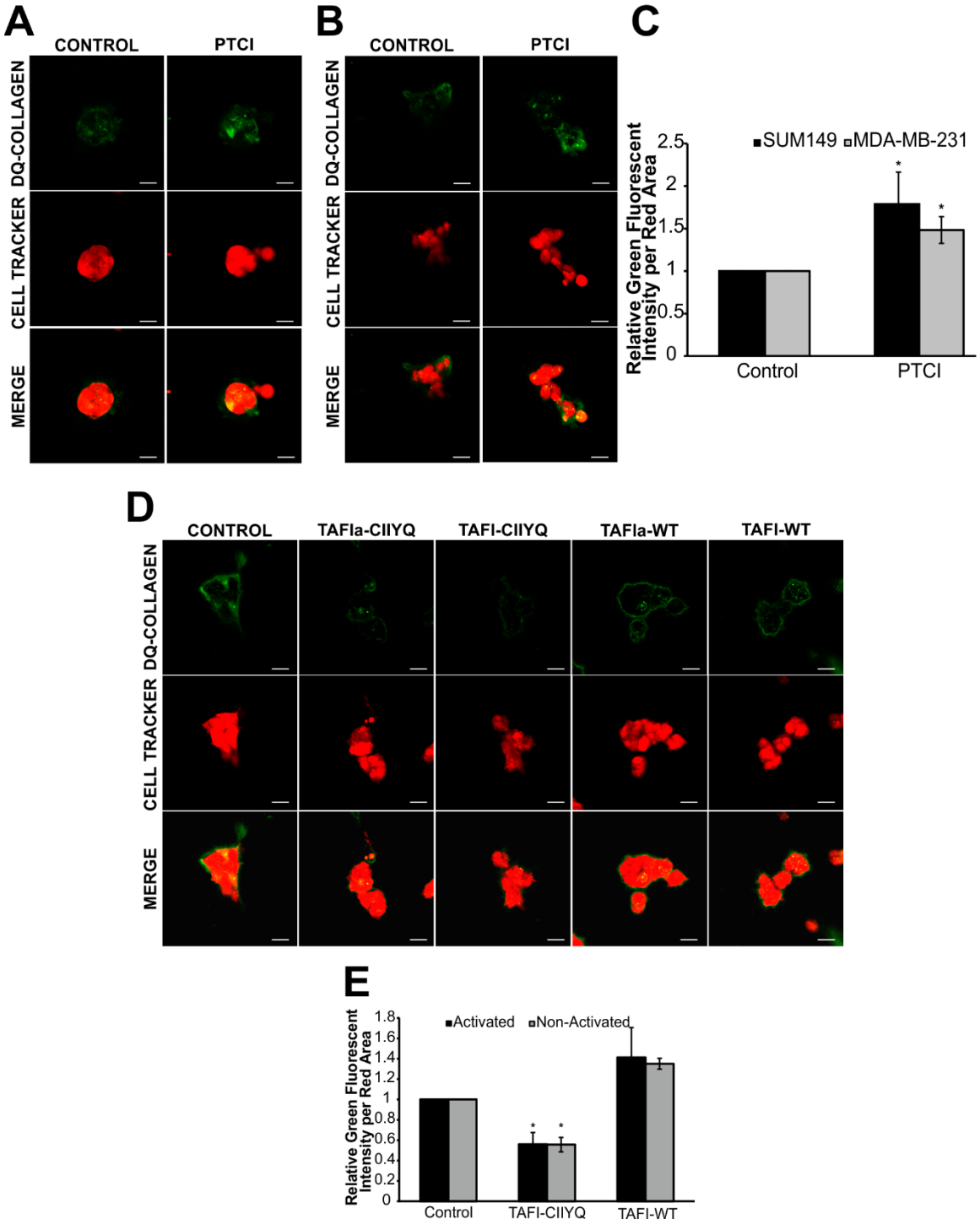
A
CONTROL
PTCI
DQ-COLLAGEN
CELL TRACKER
MERGE
B
CONTROL
PTCI
DQ-COLLAGEN
CELL TRACKER
MERGE
C
SUM149
MDA-MB-231
Relative Green Fluorescent Intensity per Red Area
2.5
2
1.5
1
0.5
0
Control
PTCI
D
CONTROL
TAFIa-CIIYQ
TAFI-CIIYQ
TAFIa-WT
TAFI-WT
DQ-COLLAGEN
CELL TRACKER
MERGE
E
Activated
Non-Activated
Relative Green Fluorescent Intensity per Red Area
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
TAFI-CIIYQ
TAFI-WT

Figure 2.6: Inhibition of TAFIa increases proteolysis of DQ-collagen type IV by breast cancer cells.

SUM149 (A) and MDA-MB-231 (B) cells labeled with CellTracker Orange (shown in red) were grown on reconstituted basement membrane extract containing 25 µg/mL DQ-collagen IV. Cells were treated with 10 µg/mL PTCI for 48 hours. Confocal images were taken following the 48-hour treatment. DQ-collagen degradation products were observed in green. Scale bars: 20 µm. (C) DQ-collagen fluorescence was quantified and normalized to the area of CellTracker Orange fluorescence. The data are represented as the green fluorescent intensity per area of the red fluorescence, averaged across the entire z-stack and expressed relative to untreated cells (Control). The data represent the mean ± SEM of 3 independent experiments, each carried out in triplicate. *: $p < 0.05$ versus Control. (D) MDA-MB-231 cells labeled with CellTracker Orange (shown in red) were grown on rBM containing 25 µg/mL DQ-collagen IV and treated with 50 nM of either TAFIa-WT or TAFIa-CIIYQ, or with 50 nM mock-activated TAFI-WT or TAFI-CIIYQ. Confocal images of cells (red) and DQ-collagen IV degradation products (green) were captured after 48 hrs. Scale bars: 20 µm. (E) Degradation of DQ-collagen IV was quantified as in (C). The data represent the mean ± SEM of 3 independent experiments, each carried out in triplicate. *: $p < 0.05$ versus Control.

2.5 Discussion

TM has been shown to be an anti-metastatic factor in animal studies [19, 31] and low levels of tumor TM have been shown to be a negative prognostic indicator in breast cancer [20]. The anti-metastatic function of TM has been shown to reside in its ability to bind to thrombin [19]. We report here that a molecular target of the thrombin TM complex – namely, TAFI – can inhibit pro-metastatic behaviours of breast cancer cells *in vitro* including invasion, migration, and extracellular proteolysis.

The thrombin-TM complex activates TAFI over 1000-fold more efficiently than thrombin alone, and 20-fold more efficiently than plasmin [32]. Accordingly, we would hypothesize thrombin-TM to be a relevant activator of TAFI in the tumor microenvironment, particularly in the absence of a strong thrombogenic stimulus that would result in generation of large amounts of thrombin and plasmin. We found that the more malignant cell lines tended to express less mRNA encoding TM (Figure 1), and hence these cells would be less able to support TAFI activation. In other words, the more malignant cell lines are characterized by a relatively low capacity to generate potentially anti-metastatic TAFIa. Indeed, although Reijerkerk and coworkers found that absence of TAFI did not affect the growth and metastasis of B16 melanoma or Lewis lung carcinoma cell lines in mice, these cell lines are unable to support TAFI activation [33].

Although we were able to detect expression of mRNA encoding TAFI in breast cancer cells, there was no apparent correlation with malignancy (Figure 1). This observation evokes the concept that the limiting factor in the anti-metastatic TAFI pathway may not be TAFI itself, but instead the presence of TM that is required for TAFI activation

in this milieu. Indeed, several studies have shown that plasma TAFI concentrations are elevated in breast cancer patients and are positively correlated with endpoints such as poor prognosis and risk of recurrence [34-36]. These findings are the opposite of what would be expected given the ability of TAFIa to inhibit pro-metastatic behaviours such as invasion that we have documented in this study. Moreover, a common single nucleotide polymorphism in *CPB2* encoding a more stable TAFIa species was also more frequent in breast cancer patients than controls [35, 36]. In the breast cancer microenvironment, TM may be provided by the breast cancer cells and/or by stromal cells such as endothelial cells and macrophages [37]. TAFI may arise from tumor cells or macrophages, but more likely from blood plasma present due to leaky tumor microvasculature [38].

We showed that TAFI can inhibit plasminogen activation on the breast cancer cell surface (Figure 2). Carboxyl-terminal lysine binding sites on cell surface receptors play a crucial role in plasminogen activation [39]. TAFIa cleaves carboxyl-terminal lysine residues from various protein and peptide substrates. Plasmin has a series of pro-metastatic effects including direct proteolysis of ECM and basement membrane proteins, activation of latent pro-MMPs, and liberation of latent growth factors from the extracellular matrix [40]. The decrease in plasmin generation in the presence of TAFIa likely results in decreased activation of latent MMPs in the extracellular milieu, amplifying the effect of TAFIa. It is notable that inhibition of TAFIa had a greater effect on cell invasion than cell migration (Figures 3,4). The former requires cells to degrade the matrix while the latter does not, and is an index merely of how fast cells can move. While the PAS has been shown to be important for cell movement [40], our data suggest a key role for TAFI in blocking proteolysis of collagen and other extracellular matrix components.

TAFIa activity is regulated by the intrinsic instability of the enzyme. While the TAFI zymogen is stable, the enzyme undergoes a spontaneous conformational change that causes loss of activity [21, 41]. The half-life of TAFIa at body temperature is 8 – 15 minutes [42]. When we added purified recombinant TAFIa to our invasion assays, we saw no effect (Figure 3). This is not surprising as the invasion assay is conducted over 24 hours and most of the TAFIa activity would have disappeared within the first hour. Indeed, when we used a stable TAFIa variant (TAFIa-CIIYQ) with a much longer half-life (1140 min [43]) we did observe a dose-dependent decrease in cell invasion (Figure 3). Addition of the non-activated forms of wild-type TAFI or TAFI-CIIYQ had no effect on invasion (Figure 3), indicating that TAFI zymogen concentration was not a limiting factor under these conditions. However, in cell migration assays, both enzymes appeared to have an effect (Figure 4), although the decreases caused by wild-type TAFIa were non-significant. Again, addition of the zymogens had no effect (Figure 4). The migration assays may have been influenced at an early stage by the presence of TAFIa, for example if most of the migration occurred in the first hours of the 24-hour incubation. Assays of the metabolic activity of the breast cancer cell lines showed minimal impact of manipulation of TAFIa activity (Figure 5). These findings suggest that the effect of TAFIa on invasion and migration cannot be attributed to changes in cell proliferation.

Inhibition of TAFIa enhanced DQ-collagen degradation, indicating an important role for TAFIa in mediating collagen proteolysis (Figure 6). Unlike cell migration, in the proteolysis assays both the wild-type and mutant zymogens of TAFI along with their active counterparts resulted in a decrease in collagen degradation (Figure 6). The 48-hour incubation for the proteolysis assays may have allowed for ongoing activation of the added

TAFI to have manifested in less accumulation of degraded DQ-collagen type IV. In the breast tumor microenvironment, we would expect continuous activation of TAFI through the action on small amounts of thrombin generated *in situ* interacting with TM on the surface of different cell types in this environment. We observed the presence of DQ-collagen type IV proteolysis both extracellularly and intracellularly (Figure 6). The proteases and pathways involved in pericellular proteolysis versus intracellular proteolysis are distinct [44, 45]. Pericellular proteolysis involves MMPs, serine proteases (including components of the plasminogen activation system), and cysteine proteases [44, 45]. Intracellular proteolysis involves endocytosis of DQ-collagen IV by the live cells followed by degradation of the substrate in the lysosome [45-47]. Intracellular proteolysis can also occur after partial degradation of DQ-collagen type IV by pericellular proteases with subsequent endocytosis and further degradation in the lysosome [48]. As such, an increase in intracellular proteolysis also reflects the extent of pericellular proteolysis. We saw effects of manipulation of TAFIa activity on both pericellular and intracellular proteolysis (Figure 6), but since TAFIa acts outside the cell, we would argue that TAFIa directly affects pericellular proteolysis, with an indirect effect on intracellular proteolysis.

In conclusion, we have demonstrated for the first time that activation of TAFI results in a suppression of breast cancer cell invasion and migration. Our data are consistent with the idea that TAFIa mediates these effects by inhibiting pericellular plasminogen activation and by attenuating proteolysis of collagen type IV. Further work will be required to define the full spectrum of the effects of TAFIa on specific proteases and protease targets in the breast tumour microenvironment. Our results indicate that promotion of TAFI activation

in this microenvironment, either by provision of soluble TM or stabilization of TAFIa, would have anti-metastatic effects.

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Chapter 3

Activated Thrombin-Activatable Fibrinolysis Inhibitor Attenuates the Angiogenic Potential of Endothelial Cells: Potential Relevance to the Breast Tumour Microenvironment

3.1 Summary

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a basic carboxypeptidase zymogen present in blood plasma. Proteolytic activation of TAFI by thrombin, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM), or plasmin results in an enzyme (TAFIa) that removes carboxyl-terminal lysine residues from protein and peptide substrates, including cell-surface plasminogen receptors. TAFIa is therefore capable of inhibiting plasminogen activation in the pericellular milieu. Since plasminogen activation has been linked to angiogenesis, TAFIa could therefore have anti-angiogenic properties, and indeed TAFIa has been shown to inhibit endothelial tube formation in a fibrin matrix. In this study, the TAFI pathway was manipulated by providing exogenous TAFI or TAFIa or by adding a potent and specific inhibitor of TAFIa. We found that TAFIa elicited a series of anti-angiogenic responses by endothelial cells, including decreased endothelial cell proliferation, cell invasion, cell migration, tube formation, and collagen degradation. Moreover, TAFIa decreased tube formation and proteolysis in endothelial cell culture grown alone and in co-culture with breast cancer cell lines. In accordance with these findings, inhibition of TAFIa increased secretion of matrix metalloprotease proenzymes by endothelial and breast cancer cells. Finally, treatment of endothelial cells with TAFIa significantly inhibited plasminogen activation. Taken together our results suggest a novel role for TAFI in inhibiting tumour angiogenic behaviours in breast cancer.

3.2 Background

Sustained angiogenesis is of one the hallmarks of cancer [1, 2] and is necessary for the growth of a tumour past a dormant avascular stage [3-6]. Tumour angiogenesis provides nutrients and oxygen to a tumour and also can act as an escape route allowing metastasis to occur [6, 7]. It is firmly established that highly angiogenic tumours have a higher metastatic potential than less vascular tumours [8].

The plasminogen activation system (PAS) is an important contributor to the metastatic process, including angiogenesis [9, 10]. The PAS is an enzymatic cascade responsible for cleavage and activation of plasminogen to its active form plasmin by urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) [11]. Plasminogen activation is enhanced on the cell surface by plasminogen receptors, some of which possess carboxyl-terminal lysine residues, allowing for plasminogen binding [12]. Plasmin is a serine protease that cleaves a wide range of substrates, including several matrix metalloproteinases (MMPs) and a variety of extracellular matrix (ECM) proteins [13], events that stimulate cell invasion, migration, and ultimately sprouting during angiogenesis [14].

Thrombin-activatable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase B2 and procarboxypeptidase U, is a plasma zymogen that plays a role in attenuating fibrinolysis [15] and in regulating pericellular plasminogen activation [16]. TAFI is activated by thrombin, plasmin and, most efficiently, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM). TAFIa is a basic carboxypeptidase that cleaves carboxyl-terminal lysine residues from various protein and peptide substrates, including fibrin degradation products and plasminogen receptors.

Recently, we have demonstrated that TAFIa possess anti-metastatic potential, inhibiting cell invasion, cell migration and collagen degradation of breast cancer cells, through attenuation of plasminogen activation [17]. Likewise, Higuchi *et al.* found that TM-stimulated activation of TAFIa decreased invasion of HT1080 fibrosarcoma cells by reducing pericellular plasminogen activation [18]. A study by Reijerkerk *et al.* reported no difference in metastasis or tumour growth in TAFI knockout mice [19]. However, the cell lines used in the latter study (Lewis lung carcinoma and B16-BL melanoma) do not support TM-mediated TAFI activation on their surface; therefore, this study does not clearly rule out a role for TAFI in cancer. The role of TAFI in tumour angiogenesis has not been previously explored, although TAFIa has been shown to reduce degradation of ECM [20] and to inhibit wound healing angiogenesis in plasma clots [21]. Collectively, these findings suggest that TAFIa has the potential to play an anti-angiogenic and anti-metastatic role in cancer progression. We therefore set out to further assess the potential of TAFI to regulate angiogenesis in a setting that, unlike a plasma clot, more closely mimics the breast cancer tumour microenvironment.

3.3 Methods

3.3.1 Cell lines and cell culture

SUM149 cell line was a gift from Dr. Stephen Ethier (Barbara Ann Karmanos Cancer Institute), MDA-MB-231 cell line was purchased from Sigma, and human umbilical vein endothelial cells (HUVECs) were purchased from Lonza. SUM149 cells were grown in Dulbecco's Modified Eagles Medium: Nutrient Mixture F-12 (DMEM/F-12) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% antibiotic/antimycotic (Gibco), 10 μg/mL insulin (Sigma-Aldrich) and 0.5 μg/mL hydrocortisone (Sigma-Aldrich). MDA-MB-231 cells were supplemented with DMEM/F-12 supplemented with 10% FBS, and 1% antibiotic/antimycotic. HUVECs were supplemented with Endothelial Cell Basal Medium-2 (EBM-2) (Lonza) completed with 2% FBS (Lonza) and Endothelial Cell Growth Media-2 (EGM-2) SingleQuots Kit (Lonza). HUVECs were used at passage five for all experiments. Human embryonic kidney (HEK 293) cells were cultured in minimum essential media (MEM) (Gibco), containing 5% FBS and 1% antibiotic/antimycotic. All cells were maintained at 37°C in a humidified 95% air/5% CO_2 atmosphere.

3.3.2 TAFI purification and activation

TAFI was cloned into a mammalian expression plasmid in-frame with a carboxyl-terminal 6×His tag encoding sequence, as previously described [17]. This plasmid was stably transfected into HEK 293 cells and conditioned media (CM) was collected from cell lines grown in triple flasks (Nunc). Recombinant TAFI (rTAFI) was purified utilizing a

Ni^{2+}-Sepharose column (GE Healthcare Life Sciences), as previously described [17]. TAFI (0.8 μM) was activated to TAFIa using 25 nM thrombin (Haematologic Technologies), 100 nM thrombomodulin (Haematologic Technologies) and 5 mM $CaCl_2$ for 10 minutes at room temperature. Following incubation, thrombin was inhibited using 200 nM H-D-Phe-Pro-Arg chloromethylketone (PPAck; Calbiochem). TAFIa was then used in experimental procedures as described below, at a final concentration of 50 nM. For experiments using non-activated TAFI, the activation mixtures were assembled as described above, in the absence of thrombin. For control experiments, the activation mixtures were assembled in the absence of TAFI. Therefore, all experiments were conducted with the equivalent concentrations of added $CaCl_2$, thrombomodulin, and chloromethylketone.

3.3.3 Endothelial cell invasion and migration assays

Transwell invasion assays were used to assess the effect of inhibiting TAFIa on endothelial cell invasion. BD Falcon cell culture inserts (8 μm pore size; PET track-etched membranes) (BD Biosciences) were coated with 2 mg/mL Cultrex® Basement Membrane Extract (BME) without Phenol Red (Trevigen) in a 24-well plate for one hour. BME was diluted to 2 mg/mL from a stock solution with serum and supplement-free EBM-2 medium (Lonza). Following incubation, the inserts were washed three times with HEPES Buffered Saline Solution (HBSS) (Lonza). Supplement free EBM-2 containing 0.5% FBS was added to the bottom of each well in the 24-well plate. Test substances added to this medium were 50 ng/mL vascular endothelial growth factor (VEGF) or 25 μg/mL potato tuber carboxypeptidase inhibitor (PTCI). For experiments using TAFI, medium in the bottom chamber contained 50 ng/mL VEGF and 50 nM activated or non-activated TAFI, or no

added TAFI. Coated inserts were placed in the wells and 50,000 HUVECs/well in 100 µL of serum- and supplement-free EBM-2 were added into the coated inserts. Cells were allowed to invade into the BME for 20 hours, after which non-invaded cells were removed with a cotton swab, and the invaded cells were fixed in methanol. The invaded cells were stained with 0.25% (w/v) Crystal Violet (Sigma-Aldrich). The cells in five different fields of view were counted under a 20× objective using an Olympus CKX415F2 Inverted Microscope.

Cell migration assays were carried out in the same manner as the cell invasion assay, except cell culture inserts were not coated with BME.

3.3.4 Cell metabolism assay

Cell proliferation of HUVECs was determined using the WST-1 assay (Roche Diagnostics), which measures metabolic activity of viable cells. HUVECs were seeded in a 96-well plate at 10,000 cells/well complete EBM-2 media. Cells were treated in quadruplicate with either 50 ng/mL VEGF or 25 µg/mL PTCI. For experiments with TAFI, cells were treated with 50 nM of either activated or non-activated TAFI, in the presence or absence of 50 ng/mL VEGF. Cells were treated for 24 hours, following which WST-1 was added to each well and incubated for 2 hours at 37°C. The cleavage of WST-1 to formazan by metabolically active cells was measured at 450 nm using a SpectraMax Plus384 Absorbance Microplate Reader (Molecular Devices).

3.3.5 Tube formation assays

In preparation for tube formation assays, 35 mm glass bottom culture dishes (MatTek) were coated with BME and incubated for 10 minutes at 37°C to allow the BME to solidify. HUVECs grown in T25 flasks to ~80% confluency were washed four times with HBSS (Lonza), and labelled with 5 μM Cell Tracker™ Orange CMTMR (Life Technologies Inc.) in EBM-2 with 0.75% FBS for one hour at 37°C. Labelled cells were then washed four times with HBSS (Lonza) and incubated in complete EBM-2 for one hour at 37°C. Following incubation, 20,000 HUVECs in EBM-2 with 0.75% FBS were seeded on top of the BME-coated glass bottom dishes and allowed to attach for 40 minutes at 37°C. Subsequently, complete EBM-2 media containing 2% BME with and without treatment (50 ng/mL VEGF or 25 μg/mL PTCI) was added. For TAFI treatments, 50 nM of either activated or non-activated TAFI or the control activation mixture was added to complete EBM-2 containing 2% BME. Cells were incubated at 37°C for 18 hours to allow formation of tube-like structures [22, 23]. Cells were then imaged using an Olympus IX81 confocal microscope. Tube formation in eight to ten different fields of view was imaged under a 10× objective. Relative tube length was measured as the total length of all tubes within the field of view divided by total number of tubes within the field of view.

For co-culture experiments, SUM149 or MDA-MB-231 cells washed with phosphate buffered saline (PBS), and labelled with 5 μM CellTrace™ Far Red DDAO-SE (Life Technologies Inc.) in serum free DMEM/F-12 for one hour at 37°C. Labelled cells were then washed with PBS, and incubated in complete DMEM/F-12 for one hour at 37°C. Following incubation, breast cancer cells (20,000 SUM149 cells or 7,000 MDA-MB-231 cells in serum free DMEM/F-12) were seeded on top of BME coated glass bottom dishes,

and allowed to attach for 40 minutes at 37°C. Complete DMEM/F-12 media containing 2% BME was added and cells were incubated at 37°C for 24 hours. Thereafter, media was removed and 20,000 HUVECs pre-labeled with Cell Tracker™ Orange CMTMR were seeded on top of the embedded breast cancer cells. Cells were treated with either VEGF (50 ng/mL), PTCI (25 µg/mL) or 50 nM TAFI, as described above. After 18 hours of incubation at 37°C, tube formation was assessed as described above.

3.3.6 Dye-Quenched (DQ)-collagen IV proteolysis assays

Proteolysis of DQ-collagen IV by HUVECs was assessed as previously described [24, 25]. Briefly, 35 mm dishes were coated with BME containing 25 µg/ml DQ-collagen IV (Life Technologies Inc.) and incubated for 10 min at 37 °C. HUVECs were then seeded on top of the BME/DQ-collagen IV mixture as described above for the tube formation assay with and without 50 ng/mL VEGF or 25 µg/mL PTCI or 50 nM TAFI/TAFIa treatments. Cells were incubated at 37°C for 18 hours, and then imaged using an Olympus IX81 confocal microscope. Three to four *z*-stacks each containing fourteen 2.5 µm slices were imaged using a 20× objective with a zoom of three, and three adjacent slices were selected for analysis (one of which generally including the slice at the equatorial plane). The amount of proteolysis was determined using ImageJ software by calculating total green fluorescence of the DQ-collagen type IV degradation products in each the three slices divided by HUVEC area and averaging over the selected slices.

Proteolysis of DQ-collagen IV by co-cultured HUVECs and breast cancer cells was assessed as previously described [26]. BME mixed with 25 µg/ml DQ-collagen IV was used to coat a 35 mm glass bottom culture dish as described above. Breast cancer cells,

pre-labeled with CellTrace™ Far Red DDAO-SE, were seeded on top of the BME/DQ-collagen IV mixture using the same cell number, procedure and treatments as described for the co-culture tube formation assay above. Cells were incubated at 37˚C for 18 hours. Areas were HUVEC tube-like structures were in contact with a breast cancer spheroid were imaged and the extent of proteolysis measured as described above for the HUVEC monocultures.

3.3.7 Plasminogen activation assay

Plasminogen activation assays were conducted as previously described [17, 27]. Briefly, HUVECs were seeded to confluency in black, clear-bottom 96 well plates. Cells were washed twice with HBS supplemented with 0.4% (w/v) BSA (HBS-BSA). Activated TAFI (0, 10, 50 nM) was added to the cells and incubated for 30 minutes at 37˚C. Following the incubation, cells were washed two times with HBS-BSA. Plasminogen activation on the cell surface was conducted using both uPA and tPA. Plasminogen was purified from human plasma using lysine-Sepharose affinity chromatography, as previously described [28]. For the uPA experiments, a solution containing 500 nM plasminogen, 20 nM uPA (EDM Millipore) and 40 mM of the fluorogenic plasmin substrate H-D-Val-Leu-Lys-AMC (Bachem) was added to the cells. For the tPA experiments, 500 nM plasminogen, 5 nM tPA (Alteplase; Kingston General Hospital) and 40 mM H-D-Val-Leu-Lys-AMC was used. Hydrolysis of the substrate was monitored for 60 minutes at 37°C, at excitation and emission wavelengths of 370 nm and 470 nm, respectively.

3.3.8 Gelatin zymography

Subconfluent monocultures of SUM149 cells or HUVECs were treated with or without 50 ng/mL VEGF or 25 µg/mL PTCI for 24 hours. Following treatment, SUM149

cells and HUVECs were washed with PBS and then serum starved for 24 hours in serum free DMEM or EBM-2 with 0.2% FBS media, respectively. CM was collected from both cell types. Lysates from both cell types were collected by adding lysis buffer (50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA). Cell lysate protein concentration was determined using a BCA assay (Life Technologies Inc.) according to the manufacturer's instructions.

In some experiments, HUVECs grown to 60% confluence were washed with HBSS (Lonza) and CM from untreated, VEGF, or PTCI treated SUM149 cells (prepared under conditions as described above) was mixed 1:1 with complete EBM-2 medium and added to the HUVECs. Control HUVECs were treated with complete EBM-2 medium alone. Following 24 hours of incubation with SUM149 CM, the HUVECs were washed with HBSS and placed in EBM-2 media with 0.2% FBS for 24 hours. The supernatant and lysate were then collected, and lysate protein concentration was determined as described above.

MMP2 and MMP9 enzymatic activities in medium from SUM149, HUVEC, or HUVEC treated with SUM149-CM were determined by SDS-PAGE gelatin zymography as described by Mohamed *et al.* [29]. Samples were mixed with 2× SDS sample buffer and equal protein amounts (20 µg/lane of SUM149 samples, 6 µg/lane of HUVEC samples) were loaded, without heating or reducing, and subjected to SDS-PAGE on 10% polyacrylamide gels containing 1% (w/v) gelatin at 125 V for 2.5 hours at 4°C. Human recombinant MMP2 (5 ng) and MMP9 (2.5 ng) (R&D Systems) were also loaded as positive controls. Following electrophoresis, gels were incubated twice in renaturing buffer (2.5% Triton X-100) for 15 minutes. Thereafter, gels were washed twice with deionized water for 15 minutes. Gels were then placed in developing buffer (50 mM Tris-HCl pH

7.8, 5 mM $CaCl_2$, 0.05% Brij 35) and incubated overnight at 37°C. Subsequently, gels were stained for 1 hour with Coomassie blue (50% methanol, 10% acetic acid, 40% water, 0.5% Coomassie brilliant blue R-250), and destained (50% methanol, 10% acetic acid, 40% water) until clear bands of proteolytic activity appeared. Relative proteolytic activity of each sample was determined by densitometry using FluoroChem® Q imaging system software (Alpha Innotech Corporation).

3.3.9 Statistical methods

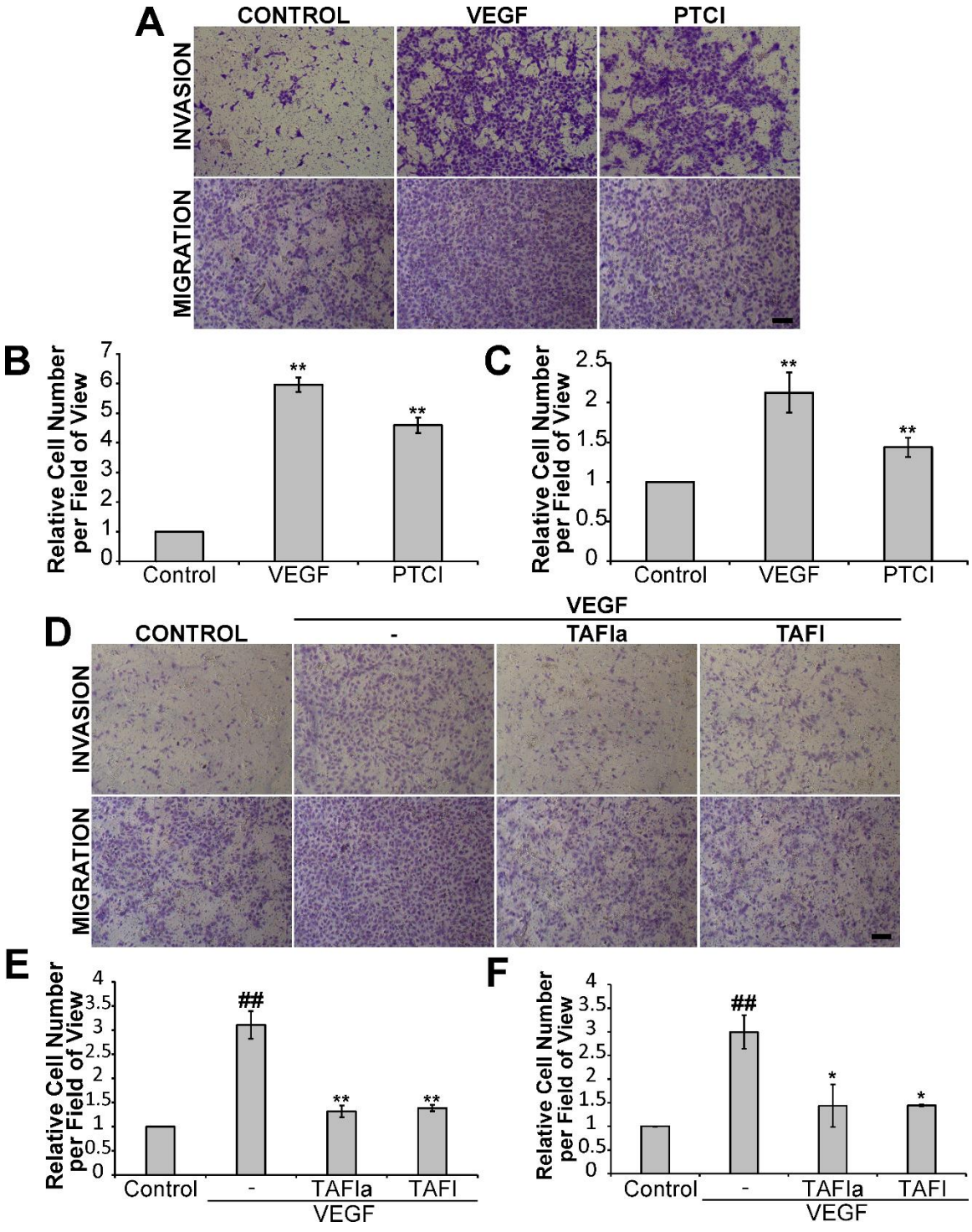
Results are expressed as a mean ± SEM from at least three independent experiments. Statistical analysis was performed using one-way ANOVA test with post-hoc Tukey HSD with IBM SPSS Statistics V22.0 software. Statistical significance was presumed at $p < 0.05$.

3.4 Results

3.4.1 TAFIa inhibits endothelial cell invasion and migration

During angiogenesis, endothelial cells locally degrade the vascular basement membrane and invade into the surrounding stroma [3, 30]. Here, we performed an *in vitro* invasion assay by seeding HUVECs in the top chamber of a BME coated Transwell insert, to assess the effect of TAFIa on endothelial cell invasion. TAFIa was inhibited using a specific competitive inhibitor, potato tuber carboxypeptidase inhibitor (PTCI). For the purposes of comparison, we also used vascular endothelial growth factor (VEGF), a known stimulator of angiogenesis. Our results show that PTCI significantly increased invasion of HUVECs 4.6-fold relative to the control, while VEGF increased invasion of HUVECs 6-fold relative to the control (Fig. 1A,B). We next assessed the direct effect of TAFI on endothelial cell invasion. Treatment with both activated and non-activated TAFI resulted in a 2.4-fold decrease in HUVEC invasion compared to the control treated with VEGF (Fig. 1E).

Cell migration is another important process that occurs during angiogenesis. Migration assays were conducted in the absence of BME and hence reflect only the motility of the cells, since there is no BME barrier that must be proteolyzed. To assess the effect of TAFI on cell migration, TAFIa was inhibited using PTCI or the cells were treated with TAFI/TAFIa. As shown in Fig. 1C, treatment with PTCI results in a significant increase in cell migration. Treatment with either activated or non-activated TAFI significantly decreased endothelial cell migration by 2.1-fold compared to VEGF alone (Fig. 1F). Together, these results demonstrate a role for TAFIa in mediating endothelial cell invasion and migration.


A
CONTROL
VEGF
PTCI
INVASION
MIGRATION
B
Relative Cell Number per Field of View
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D
CONTROL
VEGF
-
TAFIa
TAFI
INVASION
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-
TAFIa
TAFI
VEGF

Figure 3.1: TAFI decreases endothelial cell invasion and migration.
(A) HUVECs were added to a Transwell chamber coated with (for invasion), or without BME (for migration), in the presence and absence of 50 ng/mL VEGF or 25 µg/mL PTCI. Cells were allowed to invade for 20 hours and then were fixed, stained and imaged. Images above were obtained at 4× magnification. Scale bar, 200 µm **(B)** Quantification of HUVEC invasion. (**C**) Quantification of HUVEC Migration. For both cell invasion and migration, cells were counted from 5 different fields of view at 20× magnification, and cell numbers expressed relative to control. **(D)** HUVECs were added to a Transwell chamber coated with (for invasion), or without BME (for migration), in the presence 50 ng/mL VEGF with 50 nM of either activated (TAFIa) or non-activated TAFI. Cells were allowed to invade for 20 hours and then were fixed, stained and imaged. **(E)** Quantification of HUVEC invasion Panels B. **(F)** Quantification of HUVEC migration Panels C. Values are expressed as mean ± SEM from four independent experiments. ## $p < 0.01$ *versus* control, * $p<0.05$, ** $p < 0.01$ *versus* control with VEGF.

3.4.2 Effect of TAFIa on cell proliferation

We investigated whether the effects of PTCI on HUVEC invasion and migration may have been due to an increase in proliferation. After incubation for 18 hours, both VEGF and PTCI increased HUVEC metabolic activity, as measured by the WST-1 as an index of cell proliferation (Fig. 2A). We next examined the effect of TAFI and TAFIa on HUVEC metabolic activity, in the presence and absence of VEGF. In the absence of VEGF, neither TAFI nor TAFIa had a significant effect on metabolic activity (Fig. 2B). In the presence of VEGF, both the TAFI and TAFIa significantly decreased metabolic activity (Fig 2B). However, the effects of TAFIa or TAFIa inhibition were of a much lower magnitude than their effects on cell invasion and migration (Fig. 1).

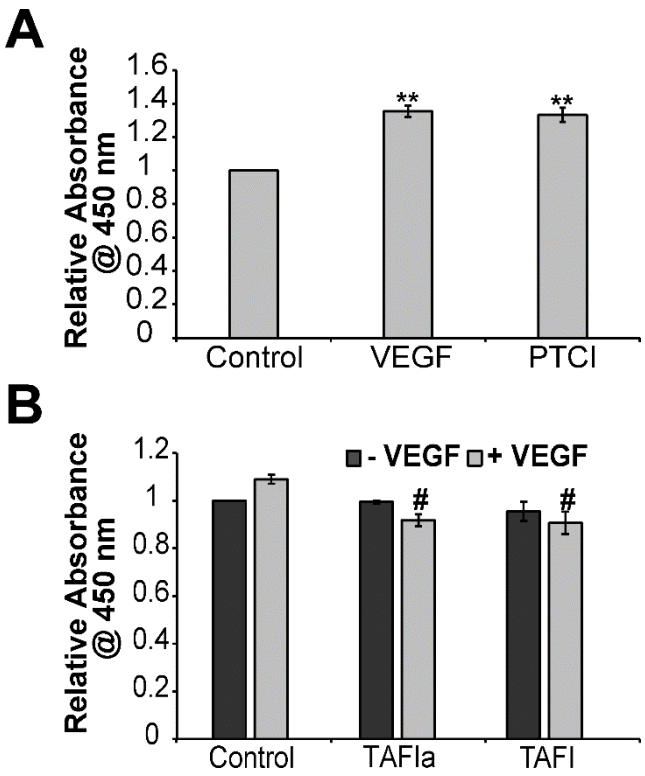

Figure 3.2: Effect of TAFI on cell proliferation of endothelial cells.
(A) HUVECs were treated for 24 hours with 50 ng/mL VEGF or 25 µg/mL PTCI. The WST-1 assay was performed as an index of proliferation. Absorbance values for the formazan product were measured at 450 nm using a plate-reading spectrophotometer. **(B)** HUVECs with treated with 50 nM of either activated or non-activated TAFI in the presence or absence of 50 ng/mL VEGF for 24 hours. Values are expressed as mean ± SEM from ≥3 independent experiments performed in quadruplicate. * $p<0.05$, ** $p< 0.01$ *versus* control, # $p<0.05$, *versus* control with VEGF.

3.4.3 TAFIa inhibits tube formation of endothelial cells grown alone and in co-culture with breast cancer cells

Another index of the angiogenic potential of endothelial cells is the formation of tube-like structures *in vitro*, in which the cells undergo a process of attachment, migration, and differentiation of endothelial cells into tube-like structures on a matrix in a manner that mimics the *in vivo* process of tube formation [23, 31]. Previous studies have shown that inhibition of TAFIa increases endothelial tube formation in a 3D plasma clot consisting of a fibrin matrix [21]. While this result demonstrated a role for TAFI in wound healing angiogenesis, the role of TAFI in endothelial tube formation during tumour angiogenesis has yet to be studied. HUVECs were seeded on top of BME, and were treated with either VEGF or the TAFIa inhibitor PTCI (Fig. 3A). PTCI promoted *in vitro* tube formation of HUVECs as observed by the significant increased relative tube length (1.3-fold) compared to the control, and resembling the effect of VEGF (Fig. 3B). We next examined the direct effect of TAFI on endothelial tube formation. Likewise, treatment of endothelial cells with both TAFI and TAFIa significantly decreased tube formation (Fig. 3C) to 0.75-fold of untreated control (Fig. 3D).

Next, we assessed the effect of TAFIa inhibition on tube formation of HUVECs grown in co-culture with either SUM149 cells (Fig. 4) or MDA-MB-231 cells (Supplementary Fig. 1), both of which are highly aggressive triple negative breast cancer cell lines. The breast cancer cells form spheroid structures on top of the BME, while the endothelial cells form tube-like structures. This *in vitro* co-culture model functions to mimic the *in vivo* tumour microenvironment, which consists of cancer cells as well as

stromal cells such as endothelial cells [32]. Interaction between HUVECs and breast cancer cells can be seen in merged images combining differential interference contrast (DIC), HUVEC fluorescence, and breast cancer cell fluorescence images (Fig. 4A/C, Supplementary Fig. 1A). Co-cultures were treated with VEGF, PTCI, TAFI, or TAFIa. PTCI significantly increased relative tube length compared to the control with a 1.4-fold increase in both the SUM149:HUVEC co-cultures (Fig. 4B) or the MDA-MB-231:HUVEC co-cultures (Supplementary Fig. 1A), and at levels similar to treatment with VEGF. Treatment with both TAFI and TAFIa decreased endothelial tube length to approximately 0.75-fold of untreated control (Fig. 4D).

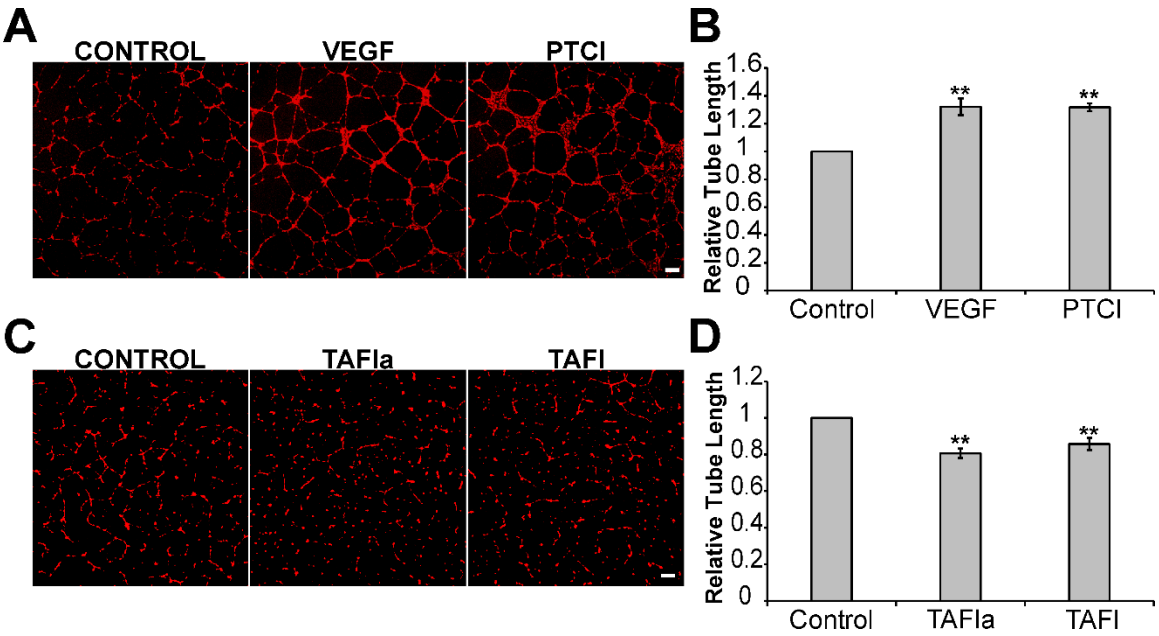

Figure 3.3: TAFIa decreases endothelial tube formation.
(A) HUVECs labelled with Cell Tracker Orange™ (red) were seeded on top of BME in a 35 mm glass bottom culture dish and treated with either 50 ng/mL VEGF or 25 µg/mL PTCI. Cells were imaged 18 hours after seeding HUVECs using confocal microscopy. Images above were obtained at 40× magnification. Scale bar, 200 µm. **(B)** Quantification of endothelial tube formation. To quantify tube formation 8-10 images were obtained at 100× magnification. Tube length was determined by measuring total tube length divided by total tube number, and is expressed relative to that in the control untreated cells. **(C)** HUVECs labelled with Cell Tracker Orange™ (red) were seeded on top of BME and treated with 50 nM of either activated or non-activated TAFI for 18 hours. Cells were imaged as in Panel A. **(D)** Quantification of tube length following treatment with TAFIa, performed as for Panel B. Values are expressed as mean ± SEM from ≥3 independent experiments. * $p<0.05$, ** $p< 0.01$ *versus* control.

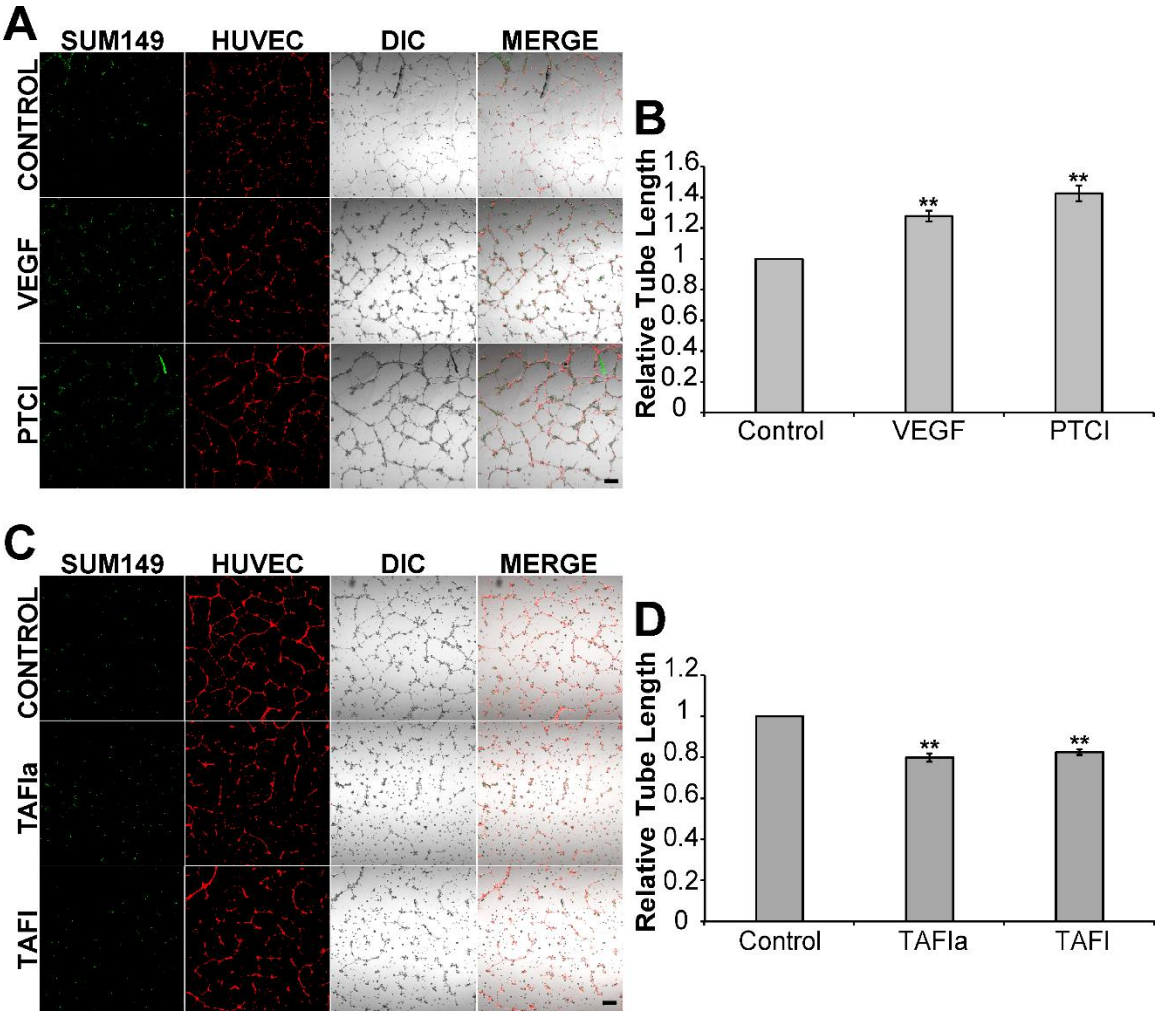

Figure 3.4: TAFIa decreases endothelial tube formation in a co-cultures of HUVECs and SUM149 cells.

(A) SUM149 cells labelled with Cell Trace™ Far Red (green) were seeded on top of BME in a 35 mm glass bottom culture dish. After 24 hours, HUVECs labelled with Cell Tracker™ Orange (red) were seeded on top of the SUM149 cells and BME. Co-cultures were treated with either 50 ng/mL VEGF or 25 µg/mL PTCI and imaged after 18 hours using confocal microscopy at 40× magnification. Scale bar, 200 µm. **(B)** To quantify tube formation, 8-10 images were obtained at 100× magnification. Average tube length was determined by measuring total tube length divided by total tube number, and is expressed relative to that in the control untreated cells. **(C)** HUVECs labelled with Cell Tracker™ Orange (red) and SUM149 cells were labelled with Cell Trace™ Far Red (blue) were seeded on top of BME and treated with 50 nM of either activated or non-activated TAFI for 18 hours. Cells were imaged as in Panel A. **(D)** Quantification of tube length following treatment with TAFI or TAFIa, performed as for Panel B. Values are expressed as mean ± SEM from ≥3 independent experiments. * $p<0.05$, ** $p< 0.01$ *versus* control.

3.4.4 TAFIa decreases proteolysis of DQ-collagen IV by endothelial cells grown alone and in co-culture with breast cancer cells

One of the initial steps of angiogenesis involves the degradation of vascular basement membrane and ECM by endothelial cells [3, 30]. Pericellular plasminogen activation has been implicated in angiogenesis and metastasis and associated with the activation of MMPs, release of growth factors from the ECM, and degradation of the ECM [33]. VEGF upregulates components of the plasminogen activation system in the leading edge of migrating endothelial cell [13, 34, 35]. Moreover, tube formation spatially correlates with ECM proteolysis *in vitro* [36]. TAFIa inhibits pericellular plasminogen activation *in vitro* and *in vivo* [37-39]. Therefore, we tested the effect of TAFIa on proteolysis of DQ-collagen IV, since collagen-IV is the most abundant type of collagen found in the basement membrane [40]. HUVECs were seeded on top of BME mixed with DQ-collagen IV, and treated with either VEGF or PTCI. Once DQ-collagen IV – a quenched fluorescent substrate – is cleaved by proteases, fluorescent DQ-collagen IV degradation products are produced (green) (Fig. 5A). Degradation products of DQ-collagen IV were localized predominantly intracellularly, although treatment with VEGF did slightly increase pericellular (outside the cell or on the cell membrane [36]) localization of DQ-collagen IV degradation products (Fig. 5A). Treatment of HUVECs with PTCI significantly increased proteolysis of DQ-collagen IV, 2-fold relative to the control (Fig. 5B). We next examined the effect of TAFI and TAFIa on DQ-collagen IV proteolysis of HUVECs (Fig. 5C). TAFIa decreased DQ-collagen IV proteolysis to 0.7-fold of control (Fig. 5D). Non-activated TAFI decreased proteolysis to 0.67-fold of control (Fig. 5D).

To examine the effect of TAFIa on proteolysis of DQ-collagen IV by both endothelial and breast cancer cells in the same environment, we co-cultured HUVECs with SUM149 cells (Fig. 6) or MDA-MB-231 cells (Supplementary Fig. 2). Co-cultures were treated with PTCI, VEGF, TAFI or TAFIa. Although both intracellular and pericellular proteolysis was observed in the co-cultures (Fig. 6A and Supplementary Fig. 2A), HUVEC:MDA-MB-231 co-cultures exhibited predominately pericellular proteolysis (Supplementary Fig. 2C). In both co-cultures, treatment with VEGF or PTCI significantly increased both pericellular and intracellular proteolysis as compared to the control (Fig. 6B and Supplementary Fig. 2D). We next examined the effect of TAFI or TAFIa treatment on proteolysis in the HUVECs: SUM149 co-cultures. Treatment with either TAFI or TAFIa resulted in a decrease in DQ-collagen proteolysis to 0.7-fold of control.

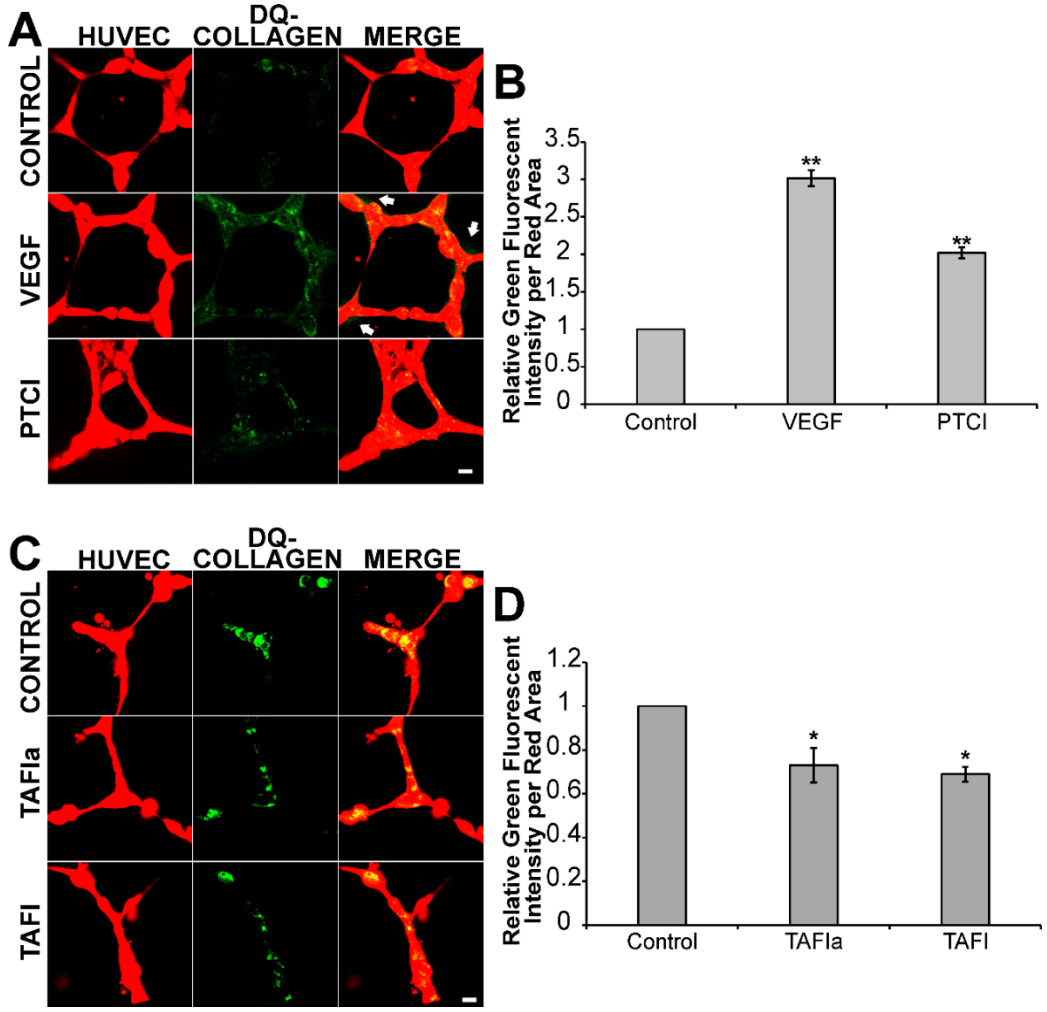

Figure 3.5: TAFIa decreases proteolysis of DQ-collagen IV by endothelial cells.
(A) HUVECs labelled with Cell Tracker™ Orange (red) were seeded on top of BME mixed with DQ-collagen IV in a 35 mm glass bottom culture dish and treated with either 50 ng/mL VEGF or 25 µg/mL PTCI. Cells were imaged 18 hours after seeding HUVECs using confocal microscopy. DQ-collagen IV cleavage products (green) were present predominantly intracellularly. Merge images show intracellular (yellow) and pericellular (green) proteolysis. Images were obtained at 600× magnification. Representative images from the equatorial plane of a Z-stack are shown. Scale bar, 20 µm. **(B)** To quantify proteolysis, three equatorial planes from 3-4 different images in each independent experiment were examined. Total integrated intensity of DQ-collagen IV fluorescence divided by total combined cell area of HUVECs was averaged across the three planes and expressed relative to the control. **(C)** HUVECs labelled with Cell Tracker Orange (red) were seeded on top of BME mixed with DQ-collagen IV and treated with 50 nM of either activated or non-activated TAFI for 18 hours. Cells were imaged as in Panel A. **(D)** Quantification of DQ-collagen proteolysis following treatment with TAFI or TAFIa, performed as for Panel B. Values are expressed as mean ± SEM from ≥3 independent experiments. * $p<0.05$, ** $p< 0.01$ *versus* control.

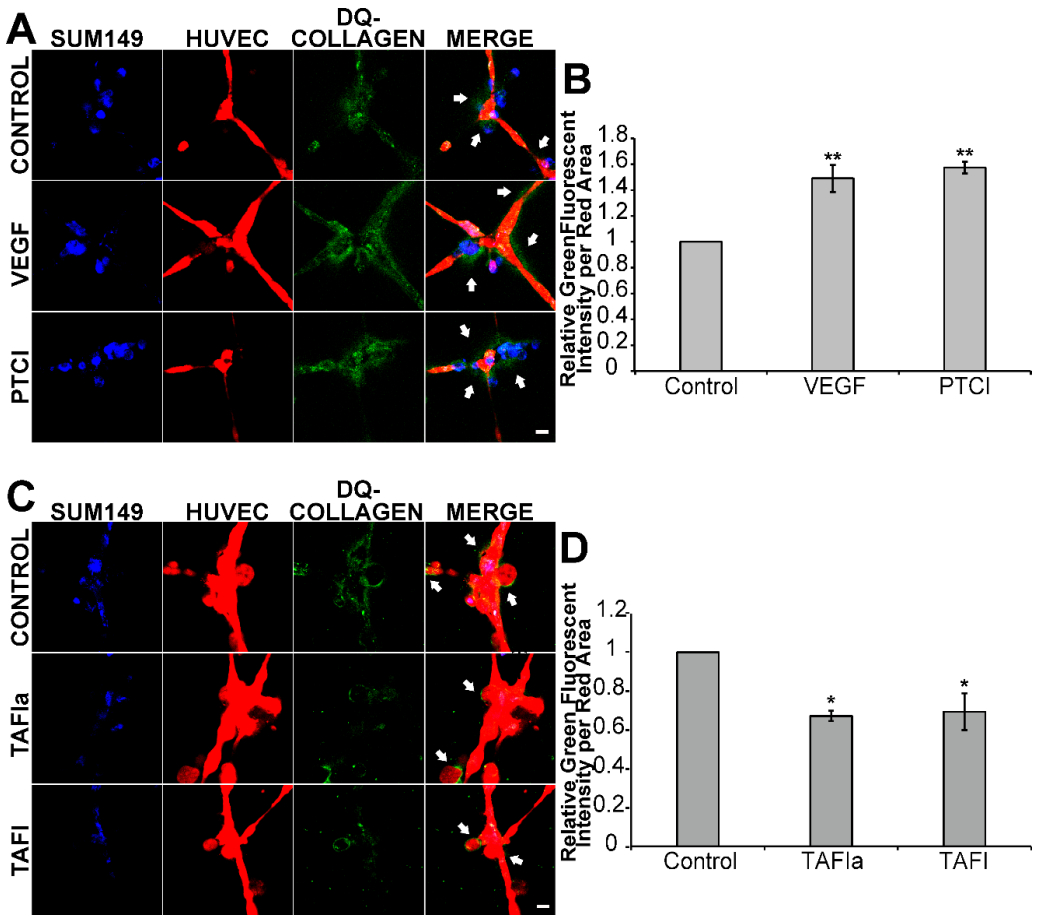

Figure 3.6: TAFIa decreases DQ-collagen IV proteolysis by co-cultured HUVECs and SUM149 cells.

(A) SUM149 cells labelled with Cell Trace Far Red (pseudocoloured blue) were seeded on top of BME mixed with DQ-collagen IV in a 35 mm glass bottom culture dish. After 24 hours HUVECs labelled with Cell Tracker Orange (red) were seeded on top of the SUM149 cells and BME mixed with DQ-collagen IV. The co-cultures were treated with either 50 ng/mL VEGF or 25 µg/mL PTCI. Cells were imaged 18 hours after HUVECs were seeded using confocal microscopy. DQ-collagen IV cleavage products (green) were present both intracellularly and pericellularly. Merged images show pericellular proteolysis (green) surrounding the co-cultured cells. Images were obtained at 600× magnification. Representative images from the equatorial plane of a Z-stack are shown. Scale bar, 20 µm. **(B)** To quantify proteolysis, three equatorial planes from 3-4 different images in each independent experiment were examined. Total integrated intensity of DQ-collagen IV fluorescence divided by total combined cell area of HUVECs and SUM149 cells was averaged across the three planes and expressed relative to the control. **(C)** HUVECs labelled with Cell Tracker Orange (red) and SUM149 cells were labelled with Cell Trace Far Red (blue) were seeded on top of BME containing DQ-collagen IV and treated with 50 nM of either activated or non-activated TAFI for 18 hours. Cells were imaged as in Panel A. **(D)** Quantification of DQ-collagen proteolysis following treatment with TAFIa, performed as for Panel B. Values are expressed as mean ± SEM from ≥3 independent experiments. * $p<0.05$, ** $p< 0.01$ *versus* control.

3.4.5 TAFIa inhibits plasminogen activation on endothelial cells

Plasminogen activation on the cell surface is accelerated by the presence of carboxyl-terminal lysine residues found on several plasminogen receptors. These lysine residues represent plasminogen binding sites on the cell surface. As a carboxypeptidase, TAFIa has been shown to cleave carboxyl-terminal lysine residues from various substrates, including plasminogen receptors. As shown in Fig. 7, various concentrations of TAFIa can inhibit both uPA and tPA mediated plasminogen activation on the surface of HUVECs. uPA-mediated plasminogen activation decreased 33% in the presence of 50 nM TAFIa; 10 nM TAFIa decreased tPA-mediated plasminogen activation 18%.

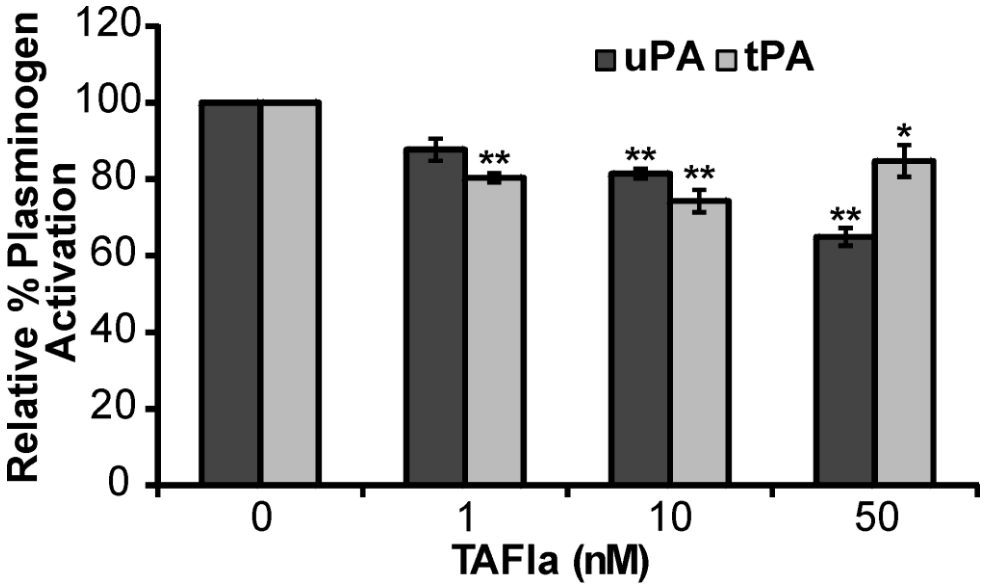

Figure 3.7: Inhibition of plasminogen activation by TAFIa on endothelial cells.
HUVECs were treated with various concentrations of TAFIa (1-50 nM) for 30 minutes at 37°C. Plasminogen activation assay was completed with 500 nM plasminogen, 20 nM uPA or 5 nM tPA and a fluorogenic plasmin substrate. The rate of plasmin generation was monitored over 1 hour at 37°C. The data are represented as the relative change in fluorescence versus time squared. The data represent results of 3-4 independent experiments and are presented as the mean ± SEM. * $p<0.05$, ** $p< 0.01$ *versus* no TAFIa.

3.4.6 Effect of inhibition of TAFIa on MMP abundance

Since inhibition of TAFIa was shown to increase proteolysis of DQ-collagen IV by endothelial cells and by endothelial cells co-cultured with breast cancer cells, we next wanted to assess whether inhibition of TAFIa affected the abundance of MMPs. Pericellular plasminogen activation is involved in ECM remodeling, as well as the release and activation of MMPs from the ECM [22]. MMP2 and MMP9 have been shown to be implicated in tumour angiogenesis [41]. MMP2 and MMP9 are gelatinases, but also degrade other ECM proteins such as collagen type IV [42]. VEGF has been shown to be in a positive feedback loop with MMP9 in RPE cells [43], therefore it was used as a positive control in these experiments.

Gelatin zymography, which allows the gelatinolytic activity of latent and active MMP2 and MMP9 to be measured [44], was used to determine the effect of inhibition of TAFIa on the abundance of MMP2 and MMP9 after treatment of SUM149 breast cancer cells with PTCI or VEGF. Bands from the SUM149 media samples migrated similarly to recombinant pro-MMP2 and pro-MMP9 controls, which suggests that they are likely pro-MMP9 and pro-MMP2 (Fig. 8A). It was determined that PTCI significantly increased abundance of both pro-MMP2 (1.7-fold) and pro-MMP9 (2.6-fold) compared to the control (Fig. 8A). We could not detect the presence of active MMP2 or MMP9 (Fig. 8A).

Similar experiments were performed to assess the effect of inhibition of TAFIa on MMP2 and MMP9 abundance in the medium of HUVECs. It was determined that PTCI does not significantly affect the abundance of pro-MMP2 (Fig. 8B). We could not detect the presence of pro-MMP9 or MMP9 (Fig. 8B).

Next, we used CM from PTCI- or VEGF-treated SUM149 cells to treat HUVECs, to examine if breast cancer cells could mediate the proteolytic activity of HUVECs in a paracrine manner. Treatment of HUVECs with CM from untreated SUM149 cells resulted in a 1.2-fold increase in pro-MMP2 abundance compared to the untreated HUVEC control, although this was not significant (Fig. 8C). Treatment of HUVECs with CM from PTCI treated SUM149 cells significantly increased the abundance of pro-MMP2, 1.6-fold relative to the untreated HUVEC control (Fig. 8C).

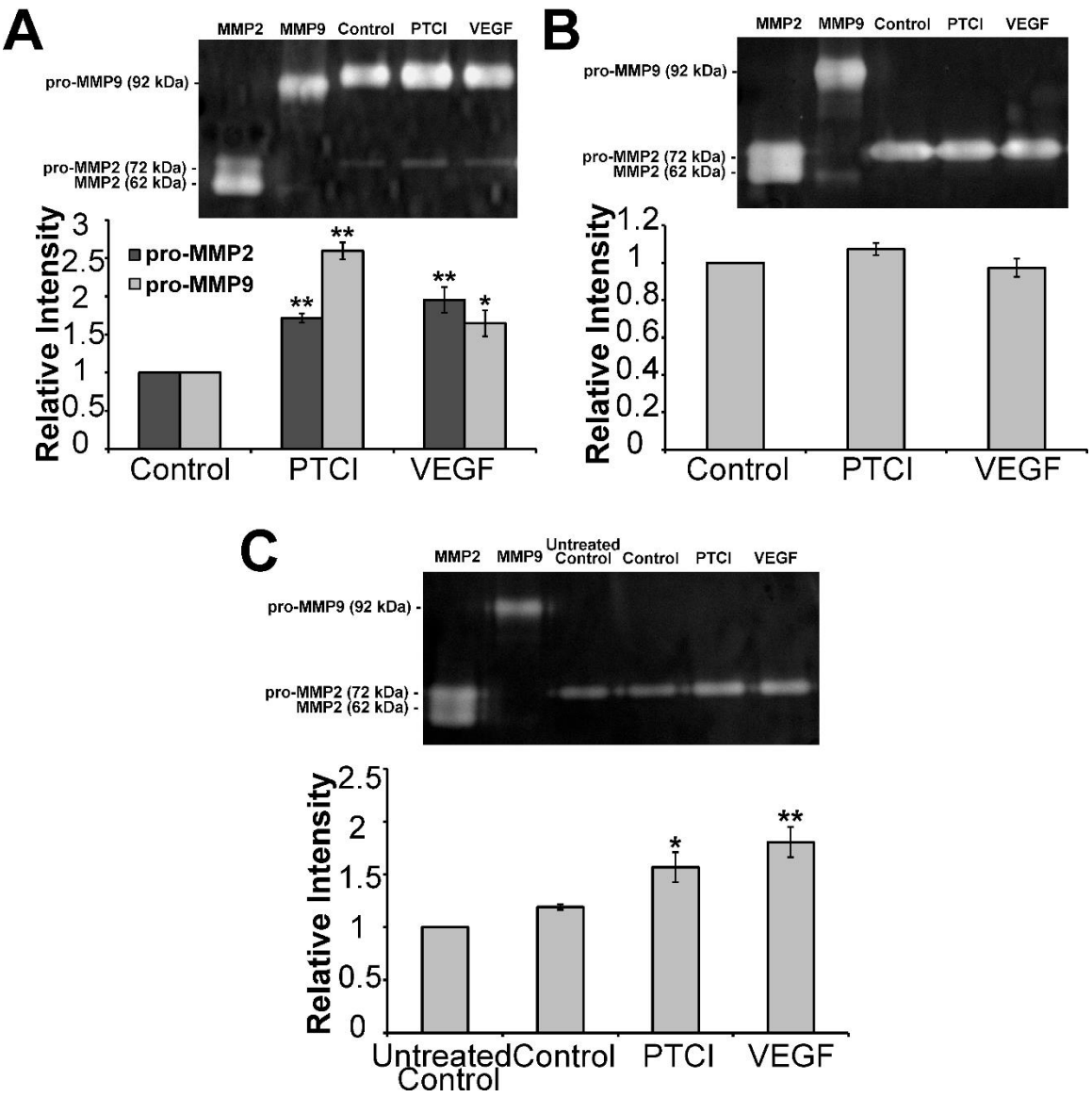

Figure 3.8: Inhibition of TAFIa in SUM149 cells increases abundance of pro-MMP2 and pro-MMP9.

(A) Representative zymogram depicting gelatinolytic activity of pro-MMP9 (92 kDa), pro-MMP2 (72 kDa) present in CM harvested from SUM149 breast cancer cells treated with either 25 µg/mL PTCI or 50 ng/mL VEGF for 24 hours. Densitometry was performed and reported as relative intensity as compared to the control. **(B)** Representative zymogram depicting gelatinolytic activity of pro-MMP2 (72 kDa) secreted by HUVECs treated with either either 25 µg/mL PTCI or 50 ng/mL VEGF for 24 hours. Densitometry was performed and reported as relative intensity as compared to the control. **(C)** Representative zymogram depicting gelatinolytic activity of pro-MMP2 (72 kDa) present in CM harvested from HUVECs treated with CM from SUM149 cells treated with either 25 µg/mL PTCI or 50 ng/mL VEGF. Densitometry was performed and reported as relative intensity as compared to the untreated control. Data represent results of ≥3 independent experiments and are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *versus* control

3.5 Discussion

Our current studies examining the role of TAFIa in angiogenic responses of endothelial cells were prompted by reports that TAFIa is able to inhibit pericellular plasminogen activation [37] and to reduce endothelial cell tube formation in a fibrin matrix [21]. We examined the role of TAFIa in angiogenesis in the context of breast cancer because we recently demonstrated that TAFIa inhibits pro-metastatic behaviours and pericellular plasminogen activation in both SUM149 and MDA-MB-231 breast cancer cells [17]. We therefore used SUM149 and MDA-MB-231 cells in co-cultures with HUVECs because they provide an appropriate cellular model to evaluate tumour angiogenesis.

Results from our *in vitro* studies suggest that TAFIa plays a role in inhibition of tumour angiogenesis. Addition of TAFI or TAFIa resulted in a decrease in crucial components of angiogenesis: namely, endothelial cell proliferation, invasion, tube formation, and ECM proteolysis, while the opposite effects were observed when endogenous TAFIa was inhibited with PTCI. Although TAFIa appeared to have a small effect on cell proliferation (Fig. 2), this is unlikely to account for the much larger effect of TAFI on cell migration and invasion, which we ascribe to effects of TAFIa on cell motility and basement membrane, respectively. Endothelial tube formation and ECM proteolysis were also decreased by TAFI/TAFIa in co-cultures of endothelial cells with breast cancer cells. We hypothesize that the ability of TAFIa to inhibit pericellular plasminogen activation likely underlies these effects, as we have shown in this study – for the first time – that TAFIa can inhibit plasminogen activation on HUVECs (Fig. 7).

For angiogenesis to occur the vascular basement membrane must be locally degraded by proteolytic enzymes [45]. ECM proteolysis promotes the angiogenic process in part by stimulating cell invasion and migration [46]. Importantly, growth factors, such as VEGF, stimulate this process [47]. In this current study we have shown that TAFIa inhibits VEGF-mediated invasion and migration of endothelial cells. Additionally, we examined the effect of TAFIa on degradation of DQ-collagen IV, a quenched fluorescent derivative of type IV collagen [48], by HUVECs grown alone and in co-culture breast cancer cells. The co-culture systems act to better recapitulate a tumour microenvironment [26]. TAFIa decreased proteolysis of DQ-collagen IV, thus supporting our hypothesis that TAFIa is able to inhibit extracellular proteolysis potentially through inhibition of plasmin formation. Indeed, pericellular proteolysis can be caused by components of the plasminogen activation system [48, 49], and plasmin can liberate and activate MMPs from the ECM [50].

Although the proteases and pathways involved in pericellular proteolysis compared to intracellular proteolysis differ in some respects [48, 49], changes in intracellular proteolysis, as we observed in some cases (Figs. 5,6) can reflect changes in extracellular proteolysis. Pericellular proteolysis have been shown to involved MMPs, serine proteases (including components of the plasminogen activation system), and cysteine proteases [48, 49]. Intracellular proteolysis involves endocytosis of DQ-collagen IV by the live cells, and degradation of the substrate in the lysosome [49-51]. Intracellular proteolysis can also occur after partial degradation of DQ-collagen IV with pericellular proteases, after which endocytosis and further degradation in the lysosome can occur [52]. In this manner the same proteolytic enzymes can be involved in pericellular and intracellular proteolysis [52].

For example, cysteine proteases degrade the DQ-collagen IV both at the cell surface and within lysosomes [48, 49].

Treatment of the HUVEC monoculture with PTCI increased intracellular proteolysis as compared to the control (Fig. 5), whereas treatment with PTCI increased pericellular proteolysis as compared to the control in both the co-culture systems (Fig. 6; Supplementary Fig. 2). Likewise, treatment with TAFI or TAFIa resulted in a decrease in intracellular proteolysis of the HUVEC monoculture, and decreased pericellular proteolysis in the co-culture system. It is not clear, however, if TAFIa has a greater effect on the breast cancer cells in terms of inhibition of pericellular proteolysis than it does on the endothelial cells, since it is unclear which cells are giving rise to the proteolytic activity in the co-cultures. Interestingly, there was a greater effect of PTCI and TAFIa in HUVEC invasion, migration and DQ-collagen proteolysis than there was in HUVEC tube formation, either in the presence or absence of breast cancer cells (compare Figs. 1, 5 and 6 to Figs. 3 and 4). This could suggest that TAFIa plays a larger role in the proteolysis and invasion aspects of angiogenesis, while other processes involved in tube formation are resistant to the effects of TAFI, such as intracellular signaling events regulating cytoskeletal remodeling or differentiation [53].

Our working model is that TAFI inhibits pericellular plasmin generation, with the consequence that there is reduced matrix and latent growth factor proteolysis by both plasmin and by plasmin-activated MMPs. Indeed, uPA bound to uPAR can enhance endothelial cell organization into tubes [54]. We looked for evidence that manipulation of TAFI did, in fact, alter levels of MMPs, using a zymography approach. We were not able to detect the cleaved, active MMP2 and MMP9 (Fig. 8), perhaps because these enzymes

are rapidly bound by inhibitors in the extracellular milieu. Intriguingly, however, we found that inhibition of TAFIa did increase the levels of pro-MMP2 and pro-MMP9 in breast cancer cell conditioned medium. Furthermore, our co-culture systems may be reporting paracrine signaling between breast cancer cells and endothelial cells [55], where CM harvested from SUM149 cells slightly increased abundance of pro-MMP2 from HUVECs (Fig. 8). Interestingly, treatment of those SUM149 cells with PTCI to inhibit TAFIa in the period prior to collection of CM significantly increased the ability of the CM to increase pro-MMP2 abundance in HUVEC CM. The mechanisms underlying these effects remain to be explored, but may relate to the effect of TAFIa on the liberation and activation of growth factors from the extracellular matrix. Whether TAFIa regulates the expression of MMP genes is also unknown at this time.

Throughout our studies, we found that addition of non-activated TAFI or TAFIa had virtually identical effects. This is in contrast to our previous study of metastatic responses by breast cancer cells [17] in which TAFIa was generally more effective than TAFI. A major difference in these studies is that HUVECs highly express thrombomodulin which is a highly effective cofactor for TAFI activation. Therefore, in the current study, it is clear that the concentration of TAFI itself is a limiting factor, whereas thrombomodulin is limiting in the breast cancer cells. Indeed, reduced tumour thrombomodulin is a negative predictor of prognosis in metastatic breast cancer [56].

In conclusion, our results show that TAFIa plays a significant role in modulating several angiogenic process, including cell invasion, migration, proteolysis and endothelial tube formation. Our findings suggest that stimulation of TAFI activation in the tumour microenvironment might be a viable strategy to inhibit tumour angiogenesis.

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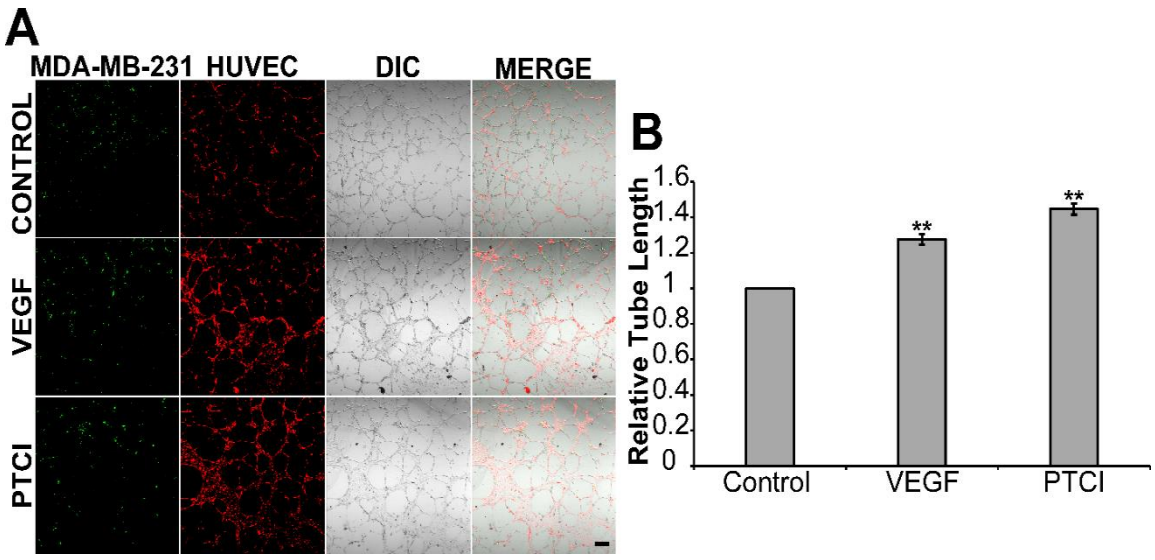
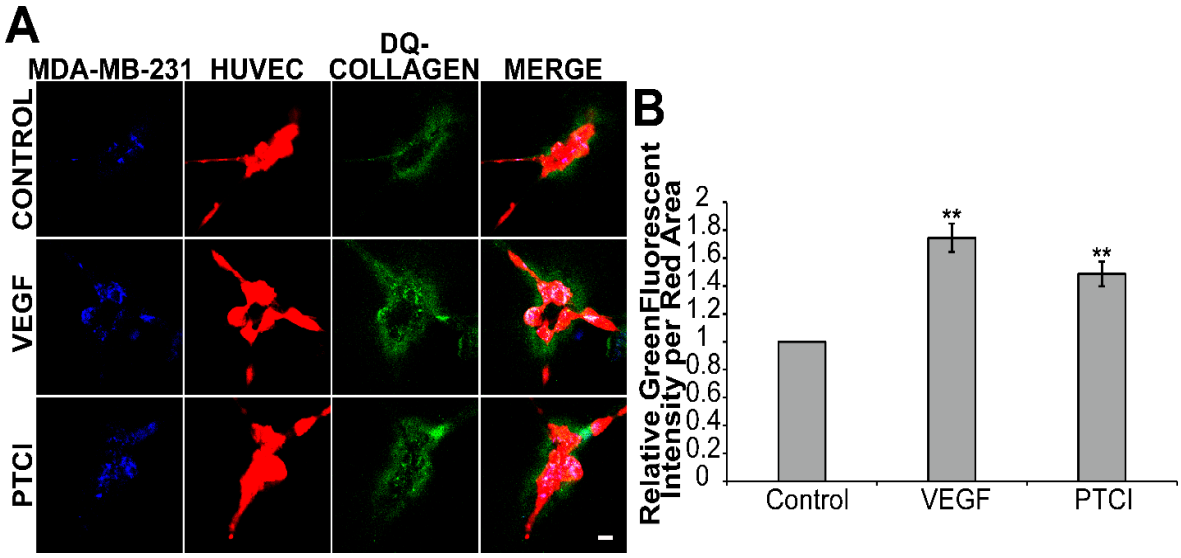
3.7 Supporting Information



Supplementary Figure 3.1: Inhibition of TAFIa increases endothelial tube formation in a co-culture system containing HUVECs and MDA-MB-231 cells.
(A) MDA-MB-231 cells labelled with Cell Trace Far Red (green) were seeded on top of BME in a 35 mm glass bottom culture dish. After 24 hours HUVECs labelled with Cell Tracker Orange (red) were seeded on top of the MDA-MB-231 cells and BME. The co-cultures were treated with either 50 ng/mL VEGF or 25 µg/mL PTCI. Cells were imaged 18 hours after HUVECs were seeded using confocal microscopy. Images above were obtained at 40× magnification. Scale bar, 200 µm. **(B)** To quantify tube formation 8-10 images were obtained at 100× magnification. Tube length was determined by measuring total tube length divided by total tube number, and is expressed relative to that in the control untreated cells. Treatment with either VEGF or PTCI significantly increased relative tube length of HUVECs. Values are expressed as mean ± SEM from six independent experiments. ** $p < 0.01$ *versus* control.



Supplementary Figure 3.2: Inhibition of TAFIa increases DQ-collagen IV proteolysis by co-cultured HUVECs and MDA-MB-231 cells.

(A) MDA-MB-231 cells labelled with Cell Trace Far Red (blue) were seeded on top of BME mixed with DQ-collagen IV in a 35 mm glass bottom culture dish. After 24 hours HUVECs labelled with Cell Tracker Orange (red) were seeded on top of the MDA-MB-231 cells and BME mixed with DQ-collagen IV. The co-cultures were treated with either 50 ng/mL VEGF or 25 µg/mL PTCI. Cells were imaged 18 hours after HUVECs were seeded using confocal microscopy. DQ-collagen IV cleavage products (green) were present predominately pericellularly. Merge images show pericellular proteolysis (green) surrounding the co-cultured cells. Images were obtained at 600× magnification. Representative images from the equatorial plane of a Z-stack are shown. Scale bar, 20 µm. **(B)** To quantify proteolysis three equatorial planes from 3-4 different images from one experiment were quantified. Total integrated intensity of DQ-collagen IV florescence divided by total combined cell area of HUVECs and MDA-MB-231 cells was averaged across the three planes and expressed relative to the control. Treatment with either VEGF or PTCI significantly increased proteolysis of DQ-collagen IV. Values are expressed as mean ± SEM from six independent experiments. ** $p < 0.01$ *versus* control.

Chapter 4

Thrombomodulin as a cofactor for TAFI and protein C activation: role in breast cancer metastatic behaviours and angiogenic potential

4.1 Summary

Metastasis is an important process in cancer progression. This process is facilitated by proteases that promote extracellular matrix degradation and cell invasion. Angiogenesis is important to the metastatic process and also involves degradation of the extracellular matrix and cell invasion. Thrombomodulin (TM) has been shown to be an anti-metastatic factor in various cell types. The anti-metastatic effects of TM have been attributed to its thrombin binding domain, which is responsible for the activation of TAFI and protein C. We therefore sought to determine which substrate of the thrombin/TM complex is important for the anti-metastatic functions of TM and what the role of TM is in angiogenesis. We examined metastatic behaviours using MDA-MB-231 and SUM149 cells. Angiogenic behaviours were tested in HUVECs and in co-culture systems with HUVECs and either MDA-MB-231 or SUM149 cells. We utilized a truncated variant of recombinant TM containing EGF-like domains 3-6 (rTM EGF306) and containing mutations that eliminated the ability of TM to promote either TAFI (V340A/D341A) or PC (F376A) activation. Treatment with rTM EGF3-6 WT increased breast cancer cell invasion and DQ-collagen proteolysis of breast cancer cells, but did not effect endothelial tube formation. Use of rTM EGF3-6 V340A/D341A consistently resulted in an increase in pro-metastatic and pro-angiogenic behaviours, including cell invasion, DQ-collagen IV proteolysis and endothelial tube formation, in all systems tested. Conversely, treatment with rTM EGF3-6 F376A decreased cancer cell invasion and DQ-collagen IV proteolysis of breast cancer cells compared to rTM EGF3-6 WT. Treatment with rTM EGF3-6 F376A also decreased endothelial tube formation and DQ-collagen IV proteolysis in HUVECs alone and in the co-culture systems, compared to control in the absence of treatment. These

results show that increasing protein C activation (using rTM EGF3-6 V340A/D341A) promotes metastatic behaviour and angiogenic potential. On the other hand, increasing TAFI activation inhibits metastatic and angiogenic potential. Together these results demonstrate that manipulating cofactor ability of TM can promote or inhibit metastatic and angiogenic behaviours.

4.2 Background

Cancer metastasis is the cause of the majority of cancer-related deaths and is characterized as the spread of cancer from the primary tumour site to a secondary location [1]. The metastatic cascade is facilitated in part by proteolytic events which degrade the extracellular matrix (ECM) and basement membrane [2]. Specifically, proteases such as plasmin and matrix metalloproteinases (MMPs) cleave ECM components, which ultimately results in cell invasion into the stromal surroundings. Proteolytic events are also important to the process of angiogenesis, the sprouting of new blood vessels from pre-existing vessels [3]. Tumour angiogenesis facilitates metastasis by providing an escape route for metastasizing cells.

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen that is activated to its active form activated TAFI (TAFIa) by cleavage by thrombin, plasmin or thrombin in complex with thrombomodulin (TM) [4-6]. TAFI activation by the thrombin/TM complex is over 1000-fold more efficient then thrombin alone, and thus is thought to represent an important physiological mode of TAFI activation [4]. TAFIa is a basic carboxypeptidase and a known inhibitor of plasminogen activation to plasmin [7, 8]. TAFIa cleaves carboxyl-terminal lysine residues from protein and peptide substrates including plasminogen receptors and fibrin degradation products [7, 8]. Carboxyl-terminal lysine residues are vital in plasminogen activation to plasmin and cleavage by TAFIa of these residues on plasminogen receptors reflects a key role for TAFI in regulation of pericellular plasminogen activation [9, 10].

TM is a transmembrane protein found on the surface of several cell types including endothelial cells. TM is comprised on a C-type lectin-like domain, 6 epidermal growth factor (EGF)-like repeats, a serine/threonine-rich domain, a transmembrane domain and a cytoplasmic tail. TM is involved in several physiological processes including inflammation and coagulation. TM has also been show to be anti-metastatic factor [11]. Expression of TM in cancer is inversely correlated to survival [12-14]. Several groups have demonstrated the important of TM in mediating cell invasion and migration of cancer cells [15-18]. Horowitz and coworkers found that the anti-metastatic effects of TM are attributed to its thrombin binding domain [19]. Importantly, binding of thrombin to TM is responsible for the activation of TAFI to TAFIa as well as the activation of protein C to activated protein C (APC). Horowitz and coworkers did not explicitly determine which substrate of the thrombin/TM complex is responsible for the anti-metastatic effects of TM. Additional studies have demonstrated that TM can act either as a pro- and anti-angiogenic factor [20, 21]. We have previously demonstrated that TAFIa inhibits metastatic behaviours of breast cancer cells by inhibition of plasminogen activation [22]. Additionally, our unpublished data have shown that TAFIa inhibits angiogenic potential of endothelial cells in breast cancer through inhibition of plasminogen activation. Conversely, there are conflicting studies with regard to the role of PC in cancer metastasis [23-26]. While some studies have demonstrated that APC is metastatic, others have shown it can act as an anti-metastatic factor. Additionally, studies have shown that APC can act as a pro-angiogenic factor [27, 28]. We therefore sought to determine which substrate of the thrombin/TM complex is responsible for the anti-metastatic effects of TM and the role of TM in tumour angiogenesis.

4.3 Methods

4.3.1 Cell Culture

MDA-MB-231, human embryonic kidney 293 cells (HEK 293) and human umbilical vein endothelial cells (HUVECs) were purchased from ATCC. SUM149 cells were a kind gift from Dr. Stephen Ethier (Karmanos Cancer Institute). MDA-MB-231 cells growth in Dulbecco's Modified Eagles Medium/ Ham's F12 (DMEM/F12) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% antibiotic/antimycotic (Gibco). HEK 293 cells were grown in minimum essential medium (MEM) (Gibco) containing 5% FBS and 1% antibiotic/antimycotic. HUVECs were cultured in Endothelial Cell Basal Medium-2 (EBM-2) (Lonza) supplemented with 2% FBS (Lonza) and Endothelial Cell Growth Media-2 (EGM-2) SingleQuots Kit (Lonza). HUVECs were used at passage five for all experiments. SUM149 cells were grown in DMEM/F12 supplemented with 5% FBS, 1% antibiotic/antimycotic, 10 μg/mL insulin (Sigma-Aldrich) and 0.5 μg/mL hydrocortisone (Sigma-Aldrich). All cells were maintained at 37˚C and 5% CO_2.

4.3.2 Construction of recombinant TM (rTM) EGF3-6

Site directed mutagenesis was completed to generate mutations corresponding to amino acid substitutions V340A/D341A and F376A within TM. Cloning vector puc19TM15 (American Type Culture Collection (ATCC)) containing the full-length TM open reading frame was used as a template and subjected to the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies), as per the manufacturer's protocol. The

primer sequences that were used for the V340A/D341A and F376A mutations are located in Table 4.1. The wild-type (WT) TM and mutated TM cDNA in puc19TM15 were then excised using *Eco*RI and inserted into pcDNA4/*myc*-His A (Invitrogen) digested with this enzyme.

Generation of rTM EGF3-6 was completed sequentially, by first removal of the serine /threonine rich domain, the transmembrane domain and the cytoplasmic domain. To do so, WT and mutant variants of TM in pcDNA4/*myc*-His A were used as a template and subjected to Q5 high fidelity polymerase (New England BioLabs), as per the manufacturer's protocol. The primers (found in Table 4.2) were used to amplify the lectin-like domain and EGF-like repeats 1-6 WT, V340A/D341A, and F376A TM in pcDNA4/*myc*-His A and introduced *Eco*RI and *Age*I sites at the 5' and 3' ends, respectively. PCR amplicons were ligated into pcDNA4/*myc*-His A following digestion with *Eco*RI and *Age*I, generating soluble TM (sTM) in pcDNA4/*myc*-His A, in frame with a 6× His-tag encoding sequence.

Following removal of domains carboxyl-terminal to EGF6, the lectin domain and EGF1-2 were removed, while maintaining the signal sequence. Briefly, QuikChange II Site-Directed Mutagenesis Kit was used to introduce *Eco*RV restrictions sites downstream of the signal peptide and upstream of EGF3. sTM variants were used as a template and both restrictions sites were introduced simultaneously, using primer pairs found in Table 4.3. Clones produced from mutagenesis were digested with *Eco*RV to remove the lectin-like domain and EGF1-2, after which the remainder was reclosed T4 DNA ligase to generate rTM EGF3-6.

Table 4.1 Primer sequences for site-directed mutagenesis of TM[a]

Mutation	Primer Sequence
V340A/D341A	5'-TAACTACGACCTG**GCG** G**CC**GGCGAGTGTGTGG-3'
F376A	5'-CTGCGCCGAGGGC**GC**CGCGCCCATTCCC-3'

[a]Only sense strand sequences are shown. Mutated codons are underlined. Mutated nucleotides are in boldface type

Table 4.2 Primer sequences for generation of sTM[b]

Primer Name	Primer Sequence
TM *Eco*RI 5'	5'-GAATTCGGCAGCGCGCAGCGGCAAGAA-3'
TM *Age*I 3'	5'-ACCGGTACAGTCGGTGCCAATGTGGCG-3'

[b]Restriction sites are underlined.

Table 4.3 Primer sequences for site-directed mutagenesis to introduce EcoRV restriction sites[c]

Primer Name	Primer Sequence
Signal Sequence	5'-CCCCGCACCCGCAGA**TATC**CAGCCGGGTGGCAGC-3'
EGF3	5'-CCCCGCACCCGCA**GATATC**CAGCCGGGTGGCAGC-3'

[c]Only sense strand sequences are shown. *Eco*RV site is underlined. Mutated nucleotides are in boldface type

4.3.3 Expression and Purification of rTM EGF3-6

rTM EGF3-6 WT, V340/D341A and F376A were stably transfected into HEK 293 cells using polyethylenimine (Sigma-Aldrich). Stably-expressing cells were selected for using a final concentration of 100 µg/mL zeocin (Life Technologies). Stably-expressing cells were grown in OPTI-MEM (Life Technologies) containing 1% antibiotic/antimycotic in roller bottle culture. Conditioned medium was harvested and subjected to Ni^{2+}-Sepharose affinity chromatography utilizing the carboxyl-terminal 6× His-tag, as previously described [22]. Following purification using Ni^{2+} affinity chromatography, protein was dialyzed against HEPES-buffered saline (HBS; 20 mM HEPES pH 7.4, 150 mM NaCl) containing 5% (v/v) glycerol and concentrated using polyethylene glycol (PEG). Following concentration with PEG, rTM EGF3-6 was subjected to further dialysis against HBS containing 5% glycerol. Protein concentrations were determined using a Pierce™ BCA Protein Assay Kit (Life Technologies Inc.) as per the manufacturer's protocol.

4.3.4 Activation of TAFI

TAFI (200 nM) was activated using 25 nM thrombin (Haematologic Technologies), 10 nM rTM EGF3-6, 5 mM $CaCl_2$ in HBST (HBS containing 0.01% (v/v) Tween 20). Control experiments were completed with rabbit lung TM (Haematologic Technologies). This is a solubilized TM that effectively accelerates activation of TAFI by thrombin and serves as an appropriate control [29]. Activation was also completed in the absence of thrombin. Activation mixture was incubated for 10 minutes at room temperature. Following the incubate, a solution containing TAFIa substrate *N*-(4-

methoxyphenylazoformyl)-Arg-OH · HCl (AAFR) (Bachem) and thrombin inhibitor D-phenylalanyl-prolyl-argininyl chloromethyl ketone (PPACK) (Calbiochem) was added to the activation mixture. Hydrolysis of AAFR by TAFIa was monitored by absorbance at 350 nm for one hour.

4.3.5 Activation of Protein C

Activation of PC was completing using 100 nM PC (Haematologic Technologies), 25 nM thrombin, 10 nM rTM EGF3-6, 5 nM $CaCl_2$ in HBS with 0.1% bovine serum albumin (BSA). Activation was also completed in the absence of thrombin. Activation mixture was incubated for 30 minutes at 37°C. Following the incubate, a solution containing 200 μM of APC substrate pyroglutamyl-prolyl-argininyl-*p*-nitroanilide hydrochloride (S-2366) (Chromogenix, Orangeburg NY, USA), and 200 nM PPACK was added to the activation mixture. Hydrolysis of S-2366 by APC was monitored by absorbance at 405 nm for one hour.

4.3.6 Cell Invasion Assay

BD Falcon cell culture inserts (8 μm pore size; PET track-etched membranes) (BD Biosciences) were coated with 2 mg/mL Cultrex Basement Membrane Extract (BME) without Phenol Red (Trevigen), for one hour at room temperature. BME was diluted in serum-free DMEM before coating inserts. DMEM/F12 with 10% FBS containing 10 nM rTM EGF3-6 WT, V340A/D41A or F376A was added the lower chamber of a 24-well plate. MDA-MDA-231 cell and SUM149 cells were diluted to a concentration of 1×10^5 cells/mL in serum-free DMEM/F12 and 5×10^4 cells were added to the upper chamber.

Cells were allowed to invade for 24 hours at 37°C. Following the incubation, non-invaded cells were removed from the upper chamber and invade cells were fixed with methanol and stained with 0.25% (w/v) crystal violet (Sigma-Aldrich). Images were taken of five fields of view and number of invaded cells were counted using ImageJ.

4.3.7 Tube Formation Assay

For tube formation assays, 35 mm glass bottom culture dishes (MatTek) were coated with BME and incubated for 10 minutes at 37°C allowing the BME to solidify. HUVECs were labelled with 5 μM Cell Tracker™ Orange CMTMR (Life Technologies Inc.) in EBM-2 with 0.75% FBS for one hour at 37°C. Following the incubation, HUVECs were washed three times with HBSS (Lonza) and allowed to recover in complete media for one hour. HUVECs were then diluted in EBM-2 with 0.75% FBS and 2×10^4 cells were added to the BME-coated culture dishes. Cells were incubated for 40 minutes to allow adhesion. Complete EBM-2 media containing 10 nM of either rTM EGF3-6 WT, V340A/D341A or F376A was then added to the dish. Cells were incubated for 18 hours to allow for tube formation. Images were taken of 8-10 fields of view using an Olympus IX81 confocal microscope. CellTracker™ Orange CMTMR was imaged at an excitation wavelength of 540 nm and an emission wavelength of 565 nm. Relative tube length was measured as the total length of all tubes within the field of view divided by total number of tubes within the field of view.

For co-culture experiments, SUM149 or MDA-MB-231 cells were labelled with 5 μM CellTrace™ Far Red DDAO-SE (Life Technologies Inc.) in serum-free DMEM/F-12 for one hour at 37°C. Following incubation, labelled cells were washed three times with

phosphate buffered saline (PBS) and allowed to recover for one hour at 37°C. Glass bottom culture dishes were coated as previously described. SUM149 or MDA-MB231 cells were diluted in serum-free DMEM/F12 and 2×10^4 cells (SUM149) or 5×10^3 cells (MDA-MB-231) were added to the BME-coated dishes. The cells were incubated for 40 minutes before complete media was added. The cells were then incubated for 24 hours, following which the tube formation protocol was carried out as previously described, with additional images taken of CellTrace™ Far Red DDAO-SE labelled cells. CellTrace™ Far Red DDAO-SE was imaged using an excitation wavelength of 630 nm and an emission wavelength of 660 nm.

4.3.8 Dye-Quenched (DQ)-Collagen IV Proteolysis Assay

MDA-MB-231 cells, SUM149 cells and HUVECs were fluorescently labelled as described above. Glass bottom dishes were coated with BME containing 25 µg/ml DQ-collagen IV (Life Technologies) for 10 minutes at 37°C. SUM149 or MDA-MB-231 cells were diluted in serum-free DMEM/F12 and 2×10^4 cells (SUM149) or $5 \text{ x } \times 10^3$ cells (MDA-MB-231) were added to the BME-coated dishes. HUVECs were diluted in EBM-2 with 0.75% FBS and 2×10^4 cells were added to the BME-coated culture dishes. Cells were allowed to adhere to the BME for 40 minutes at 37°C. Complete media containing 10 nM of either rTM EGF3-6 WT, V340A/D341A or F376A were added to the dishes. SUM149 and MDA-MB-231 cells were incubated for 48 hours at 37°C before being subjected to confocal microscopy. HUVECs were incubated for 18 hours at 37°C prior to imaging. Stacked images were taken of 3-4 fields of view. The fluorescent intensity of DQ-collagen IV (green) per cell area (red) was calculated. For experiments with HUVECs,

amount of proteolysis was determined using ImageJ software by calculating total fluorescence of the DQ-collagen type IV divided by HUVEC area for three adjacent slices (one slice at the equatorial plane and one below and above). Values of the fluorescent intensity per cell area were averaged across the three slices. For co-culture experiments, amount of proteolysis was determined using ImageJ software by calculating total fluorescence of the DQ-collagen type IV divided by the combined area of breast cancer cells and HUVECs. Three adjacent slices were used for these experiments and averaged, as mentioned above. For experiments with breast cancer cells, analysis was completed as previously described [22].

4.3.9 Statistical Methods

Results are expressed as a mean ± SEM from at least three independent experiments. Statistical analysis was performed using one-way ANOVA test with post-hoc Tukey HSD with IBM SPSS Statistics V22.0 software. Statistical significance was assumed at $p < 0.05$.

4.4 Results

4.4.1 Effect of rTM EGF3-6 mutants on breast cancer cell invasion

We sought to determine which substrate of the thrombin/TM complex is responsible for the anti-metastatic effects of TM. To do so we generated mutants of TM that can either support only TAFI activation or only PC activation. Using site-directed mutagenesis, Wang *et al.* previously determined residues of TM that are crucial for thrombin/TM-mediated activation of TAFI and PC. Mutagenesis of V340/D341A abolishes TM cofactor activity for TAFI activation. Mutagenesis of F376A inhibits activation of PC by thrombin/TM. We generated these mutants in the context of a truncated variants of rTM, EGF3-6, since the minimum essential structure of TM necessary for activation of TAFI is the c-loop of EGF-3 to EGF6 (shown in Figure 4.1). To verify the effects of the mutations in these novel rTM constructs, TAFI and PC were activated by thrombin in the presence of the rTM variants and activity assays were completed to assess activity of each enzyme. The mutants behaved as previously published and rTM EGF3-6 (Fig. 4.2) behaved similar to the TM control (data not shown).

Cell invasion is a measure of pro-metastatic behaviour and therefore we examined the effect of the rTM EGF3-6 mutants on breast cancer cell invasion. Treatment of MDA-MB-231 cells with rTM EGF3-6 resulted in a significant increase in cell invasion (Fig.4.3A, B). Conversely, treatment of SUM149 cells with rTM EGF3-6 did not affect cell invasion (Fig. 4.3C, D). Treatment of both MDA-MB-231 and SUM149 cells with rTM EGF3-6 V340A/D341A (the mutant that does not support TAFI activation) increased cell invasion (Fig. 4.3). Furthermore, rTM EGF3-6 F376A (the mutant that does not

support PC activation) significantly decreased MDA-MB-231 invasion when compared to rTM EGF3-6 WT (Fig. 4.3A, B). However, this effect was not observed in SUM149 cells (Fig. 4.3C, D). These results suggest that the thrombin cofactor ability of TM affects cell invasion.

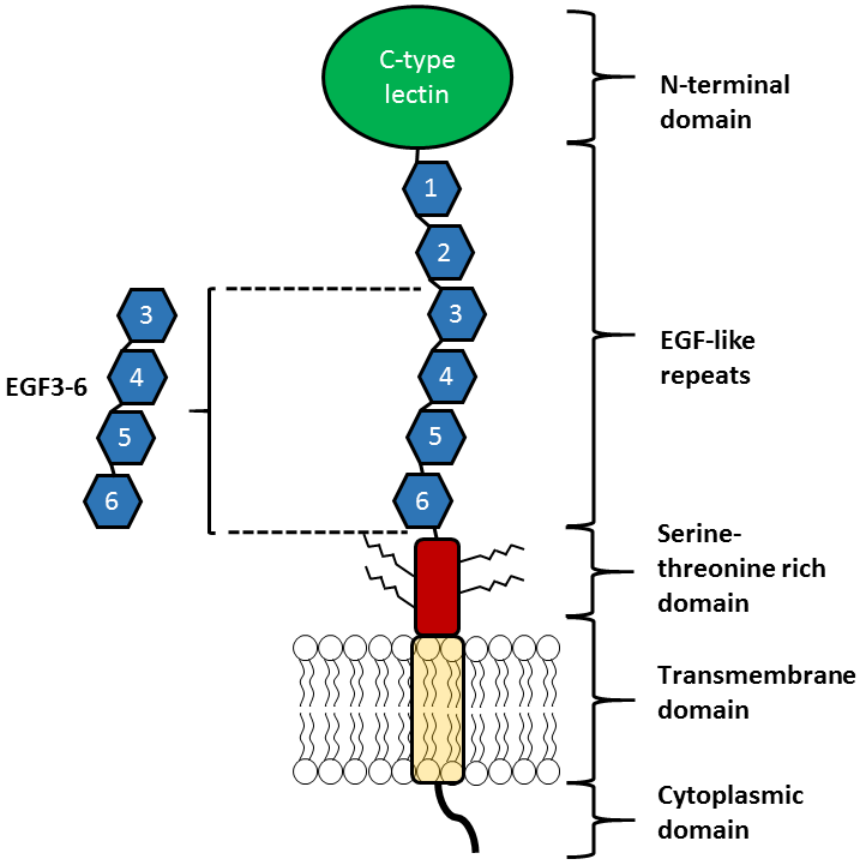


Figure 4.1: Domain structure of thrombomodulin.
Thrombomodulin contains a C-type lectin-like domain, six EGF-like repeats, a serine/threonine rich domain, a transmembrane domain and cytoplasmic tail. The EGF-like domain is responsible for the cofactor functions of TM, involving thrombin-mediated activation of TAFI and protein C. Illustrated is EGF3-6 which is the minimum necessary structure for activation of TAFI and which corresponds to the soluble TM variant employed in this study.

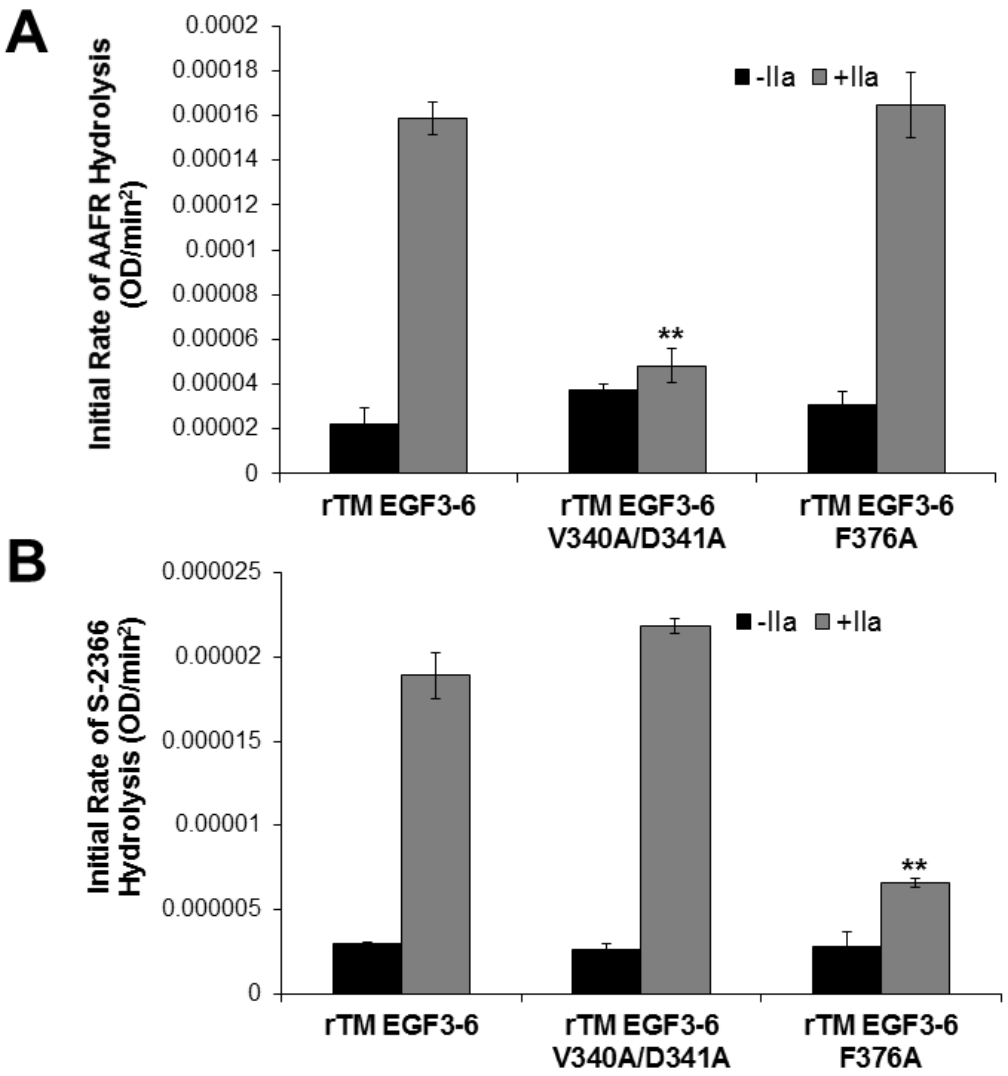


Figure 4.2: Characterization of cofactor ability of rTM EGF3-6 mutants
(A) TAFI was activated in the presence and absence of 25 nM thrombin (IIa) and in the presence of 10 nM of the rTM EGF3-6 mutants, for 10 minutes at room temperature. Following activation, thrombin was inhibited with PPACK and TAFI activity was measured by initial rate of AAFR hydrolysis. **(B)** PC was activated in the presence and absence of thrombin and in the presence of 10 nM of the rTM EGF3-6 mutants, for 30 minutes at 37°C. Following activation, thrombin was inhibited using PPACK and PC activity was measured by the initial rate of S-2366 hydrolysis. The data represent the mean ± SEM from 3-5 independent experiments. ** $p < 0.01$ relative to rTM EGF3-6.

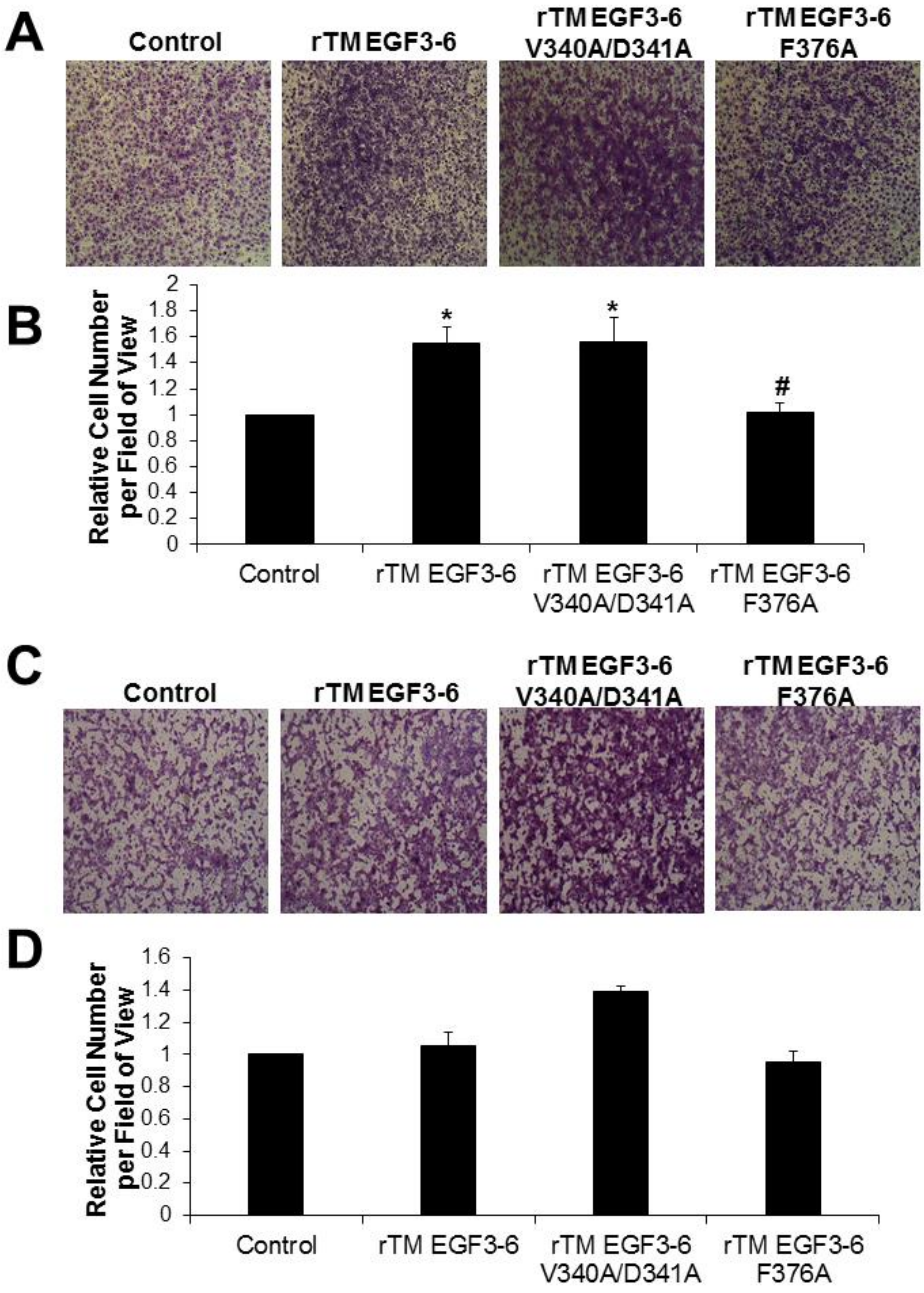


Figure 4.3: The effect of rTM EGF3-6 mutants on breast cancer cell invasion.
(A) MDA-MB-231 cells and **(C)** SUM149 cells were subjected to invasion assays in the presence of 10 nM rTM EGF3-6 mutants. The number of invaded cells were imaged and counted in five fields of view. Images shown were taken at 4× magnification. **(B)** Quantification of invaded MDA-MB-231 cells relative to control (absence of treatment). The data represent the mean cell number per field of view ± SEM from 3 independent experiments. *: $p < 0.05$ relative to control and #: $p < 0.05$, relative to rTM EGF3-6 WT. **(D)** Quantification of invaded SUM149 cells relative to control in the absence of treatment. The data represent the mean cell number per field of view 2 independent experiments.

4.4.2 Effect of rTM EGF3-6 mutants on tube formation in endothelial cells and in co-culture systems containing endothelial and breast cancer cells

There are conflicting studies on the effect of TM in angiogenesis [20, 21]. We therefore examined the effect of the rTM EGF3-6 mutants on angiogenesis, we performed an *in vitro* tube formation assay. Endothelial cells are able to form tube-like structures in the presence of BME, which mimics *in vivo* tube formation. Endothelial cells were treated with rTM EGF3-6 WT and both mutants. There was no difference in tube formation upon treatment with rTM EGF3-6 (Fig. 4.4). Treatment with rTM EGF3-6 V340A/D341A resulted in a significant increase in tube formation, when compared to the control in the absence of treatment and rTM EGF3-6 WT (Fig. 4.4). Conversely, rTM EGF3-6 F376A significantly decreased endothelial tube formation, when compared to the control in the absence of treatment and rTM EGF3-6 WT (Fig. 4.4).

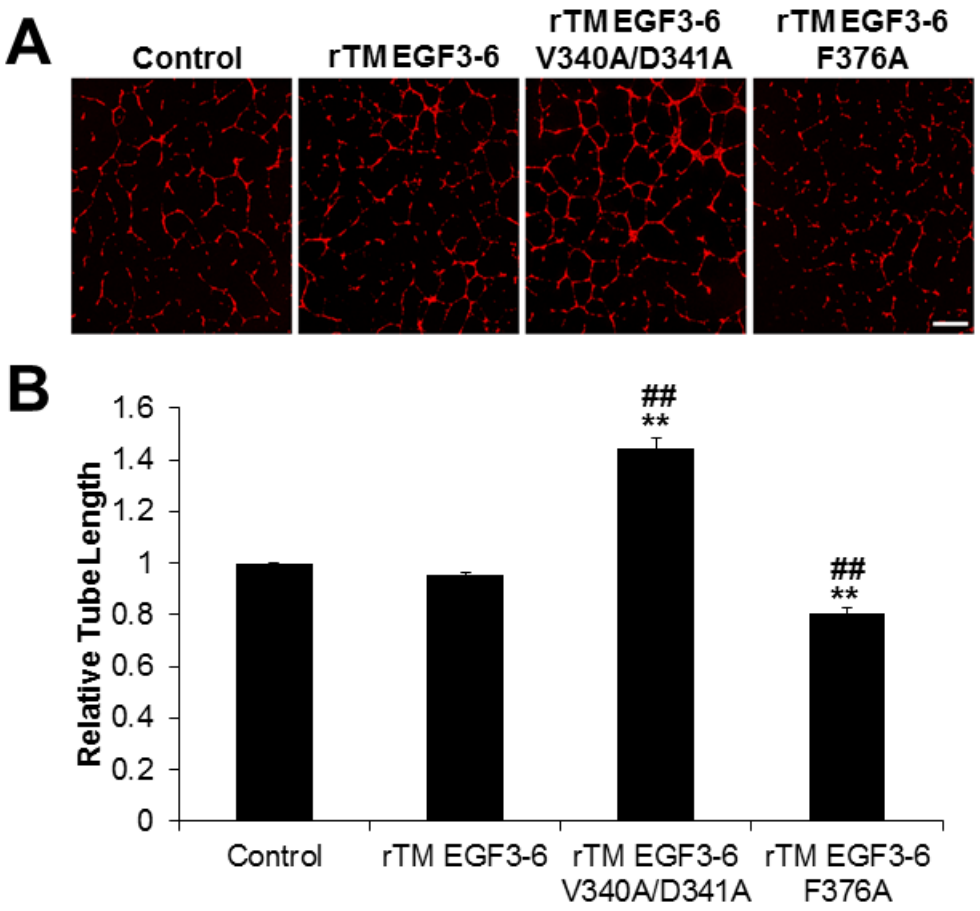


Figure 4.4: The effect of rTM EGF3-6 mutants on endothelial tube formation.
(A) HUVECs labelled with Cell Tracker Orange (red) were seeded on BME and treated with 10 nM of the rTM EGF3-6 mutants. Cells were incubated for 18 hours at 37°C and then imaged using confocal microscopy, at 40× magnification. Scale bar, 500 µm. **(B)** Quantification of endothelial tube formation. Images were taken of 8-10 fields of view. Relative tube length was obtained by measuring the total tube length per field of view divided by the total tube number per field of view. The data are represented as mean ± SEM from 6-10 independent experiments. **p< 0.01 versus control, ## p< 0.01 relative to rTM EGF3-6.

We next examined the effect of the rTM EGF3-6 mutants on endothelial tube formation in the presence of SUM149 or MDA-MB-231 breast cancer cell lines. There was no difference in tube formation in the presence of rTM EGF3-6 when compared to the control, for both MDA-MB-231 (Fig. 4.5) and SUM149 (Fig. 4.6) cells. The variant of TM that does not support activation of TAFI (rTM EGF3-6 V340A/D341A) stimulated tube formation, compared to the control in the absence of treatment and rTM EGF3-6 WT. Similar results were observed for both MDA-MB-231 (Fig. 4.5) and SUM149 (Fig. 4.6) cells. Furthermore, the variant of TM that only supports TAFI activation (rTM EGF3-6 F376A) significantly hindered endothelial tube formation, for both MDA-MB-231 (Fig. 4.5) and SUM149 (Fig. 4.6) breast cancer cell lines.

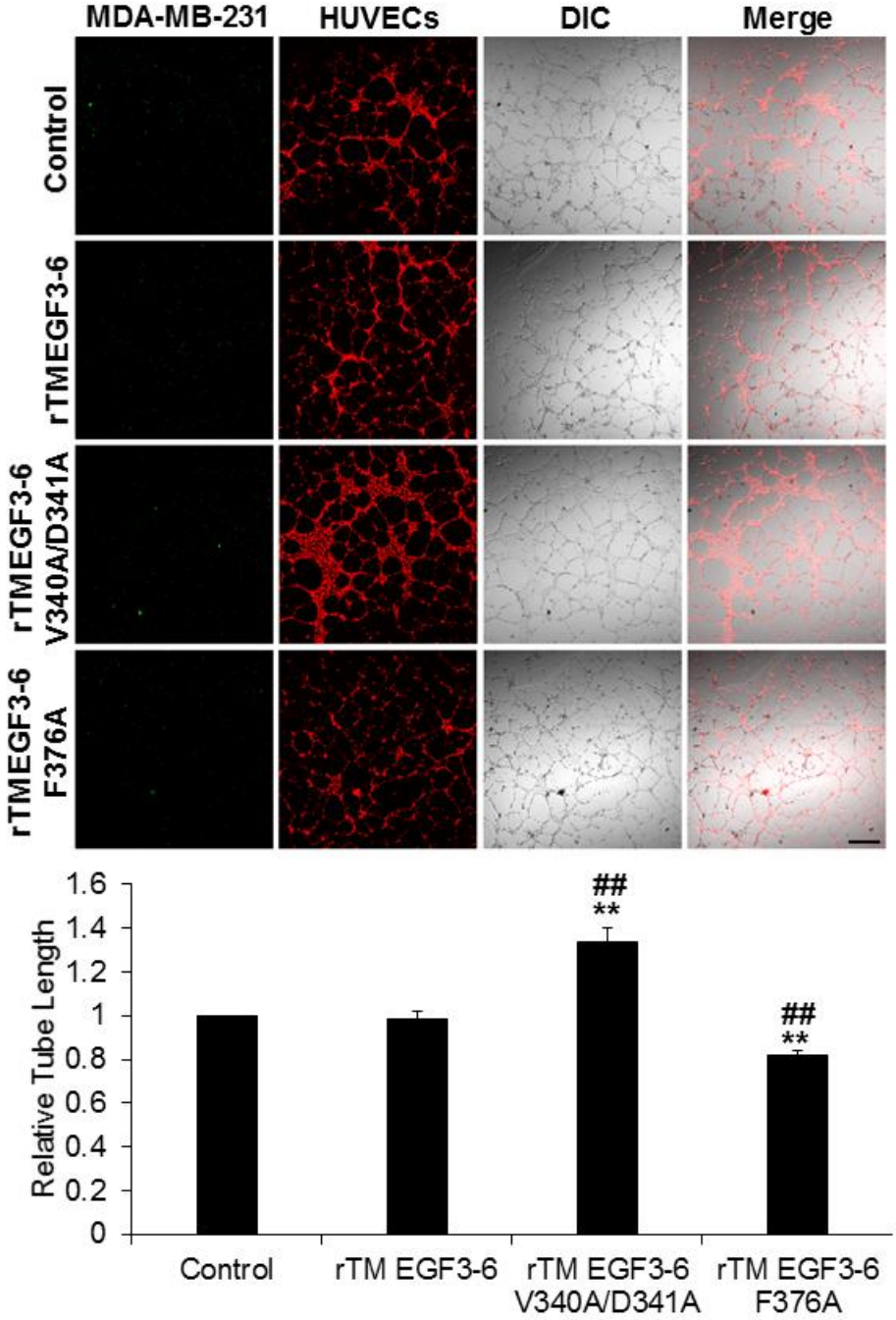

MDA-MB-231
HUVECs
DIC
Merge
Control
rTMEGF3-6
rTMEGF3-6 V340A/D341A
rTMEGF3-6 F376A
Relative Tube Length
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
##
**
##
**
Control
rTM EGF3-6
rTM EGF3-6 V340A/D341A
rTM EGF3-6 F376A

Figure 4.5: The effect of rTM EGF3-6 mutants on endothelial tube formation in a co-culture system containing HUVECs and MDA-MB-231 cells.
(A) MDA-MB-231 cells, labeled with Cell Trace Far Red (green), were seeded on BME and incubated for 24 hours before HUVECs labeled with CellTracker Orange (red) were on top of the MDA-MB-231 cells. Cells were treated with 10 nM of each of the rTM EGF3-6 variants and incubated for 18 hours prior to confocal microscopy. Images were obtained at 40× magnification. Scale bar, 500 μm. **(B)** Quantification of endothelial tube formation. Images were taken of 8-10 fields of view. Relative tube length was obtained by measuring the total tube length per field of view divided by the total tube number per field of view. The data are represented as mean ± SEM from 8-10 independent experiments. **$p < 0.01$ versus control, ## $p < 0.01$ versus rTM EGF3-6.

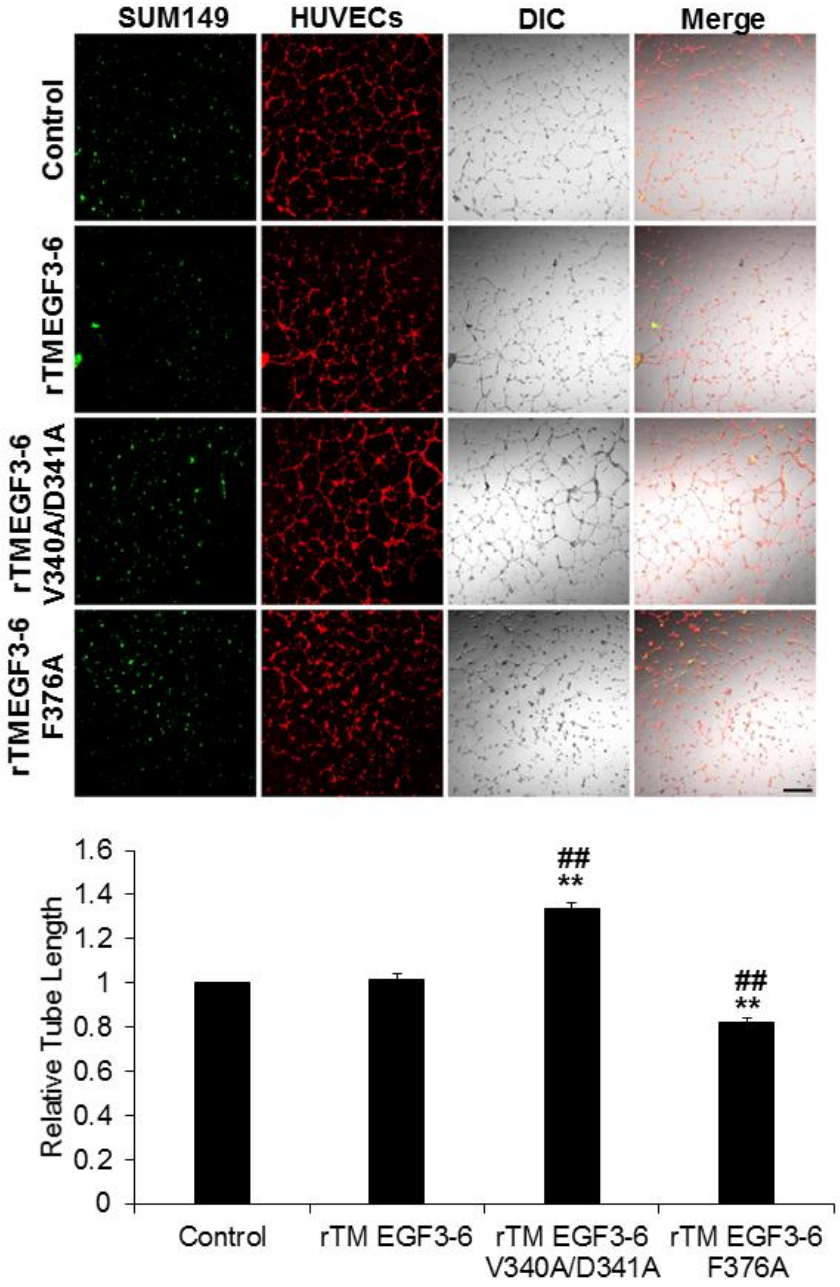

SUM149
HUVECs
DIC
Merge
Control
rTMEGF3-6
rTMEGF3-6 V340A/D341A
rTMEGF3-6 F376A
Relative Tube Length
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
##
**
##
**
Control
rTM EGF3-6
rTM EGF3-6 V340A/D341A
rTM EGF3-6 F376A

Figure 4.6: The effect of rTM EGF3-6 mutants on endothelial tube formation in a co-culture system containing HUVECs and SUM149 cells.
(A) SUM149 cells, labeled with Cell Trace Far Red (green), were seeded on BME and incubated for 24 hours before HUVECs labeled with CellTracker Orange (red) were on top of the SUM149 cells. Cells were treated with 10 nM of each of the rTM EGF3-6 variants and incubated for 18 hours prior to confocal microscopy. Images were obtained at 40× magnification. Scale bar, 500 μm. **(B)** Quantification of endothelial tube formation. Images were taken of 8-10 fields of view. Relative tube length was obtained by measuring the total tube length per field of view divided by the total tube number per field of view. The data are represented as mean ± SEM from 5-8 independent experiments. **$p < 0.01$ versus control, ## $p < 0.01$ versus rTM EGF3-6.

4.4.3 Effect of Effect of rTM EGF3-6 mutants on DQ-collagen IV proteolysis in breast cancer cells, endothelial cells and in co-culture systems containing endothelial and breast cancer cells

Extracellular proteolysis is an important step in cancer metastasis [2]. We sought to examine the effect of the TM mutants on proteolysis of DQ-collagen IV in MDA-MB-231 and SUM149 cells. Treatment with rTM EGF3-6 WT increased DQ-collagen IV proteolysis, compared to control, in MDA-MB-231 (Fig. 4.7) and SUM149 (Fig. 4.8) cells. DQ-collagen IV proteolysis also increased upon treatment with rTM EGF3-6 V340A/D341A in both MDA-MB-231 (Fig. 4.7) and SUM149 (Fig 4.8) cells. The mutant that only supports TAFI activation decreased DQ-collagen IV proteolysis in both breast cancer cell lines, when compared to rTM EGF3-6 WT (Fig. 4.7, 4.8).

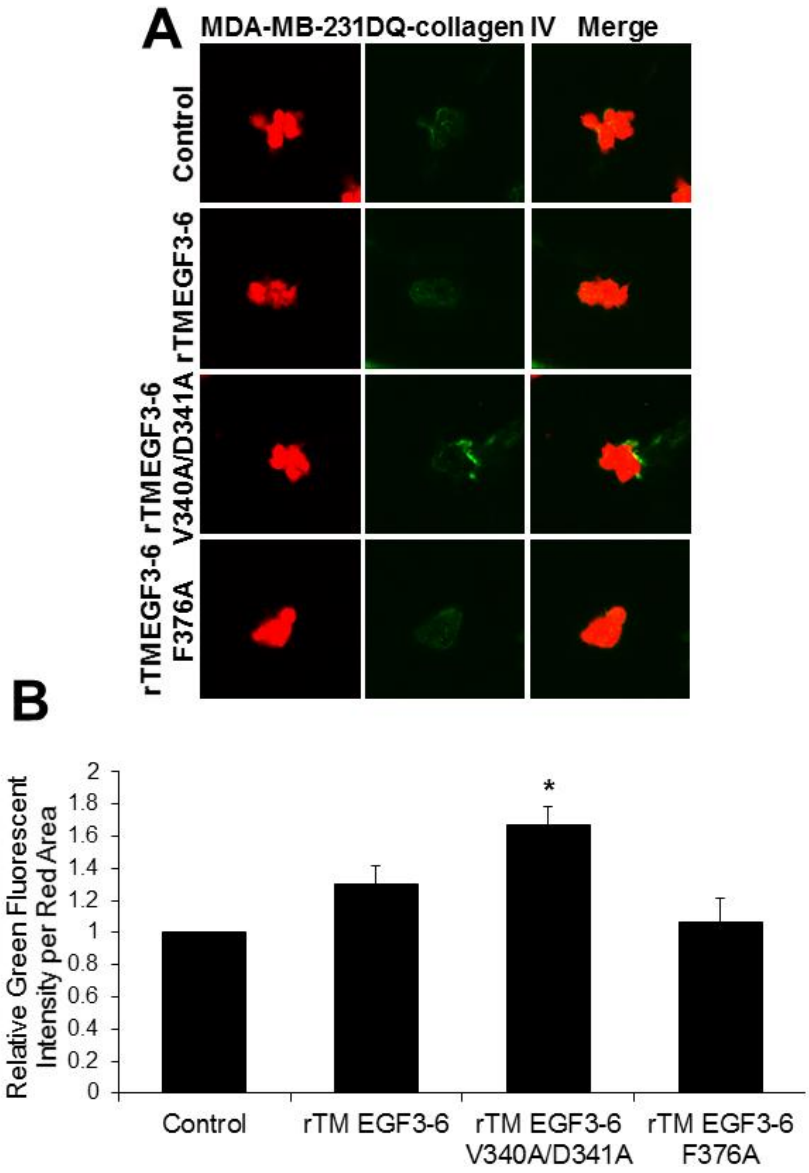


Figure 4.7: The effect of rTM EGF3-6 mutants on DQ-collagen IV proteolysis in MDA-MB-231 cells.

(A) MDA-MB-231 cells labeled with CellTracker Orange (shown in red) were grown on BME containing 25 µg/mL DQ-collagen IV. Cells were treated with 10 nM rTM EGF3-6 mutants for 48 hours. Confocal images were taken following the 48 hour incubation. DQ-collagen degradation products were observed in green. (B) DQ-collagen fluorescence was quantified and normalized to the area of CellTracker Orange fluorescence. The data are represented as the green fluorescent intensity per area of the red fluorescence, averaged across the entire z-stack and expressed relative to untreated cells (Control). The data represent the mean ± SEM of 3 independent experiments, each carried out in triplicate. *: $p < 0.05$ versus Control.

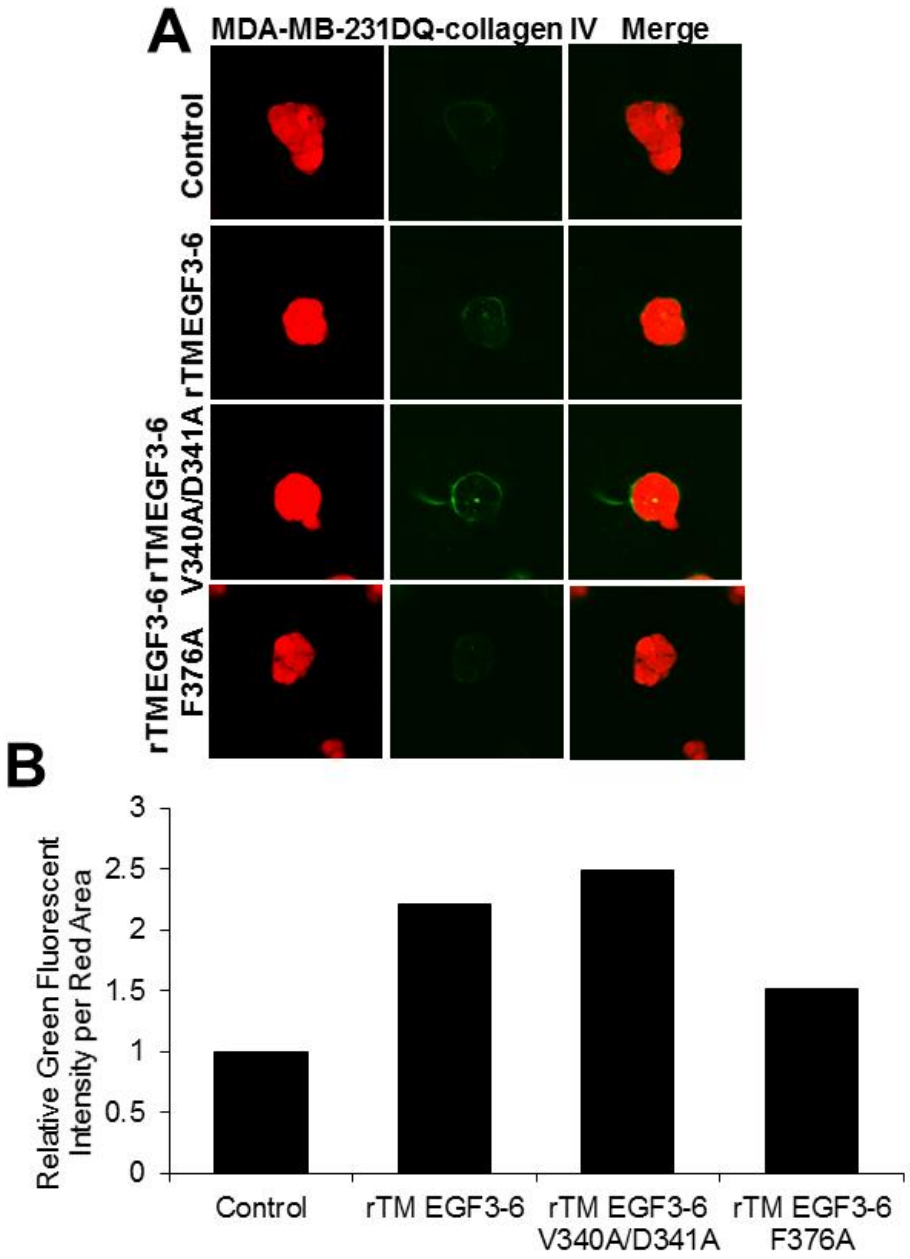


Figure 4.8: The effect of rTM EGF3-6 mutants on DQ-collagen IV proteolysis in SUM149 cells.

(A) SUM149 cells labeled with CellTracker Orange (shown in red) were grown on BME containing 25 µg/mL DQ-collagen IV. Cells were treated with 10 nM rTM EGF3-6 mutants for 48 hours. Confocal images were taken following the 48-hour incubation. DQ-collagen degradation products were observed in green. (B) DQ-collagen fluorescence was quantified and normalized to the area of CellTracker Orange fluorescence. The data are represented as the green fluorescent intensity per area of the red fluorescence, averaged across the entire z-stack and expressed relative to untreated cells (Control). The data represent 1 experiment, carried out in triplicate.

Extracellular proteolysis is also important in tumour angiogenesis [3]. We examined the effect of the TM mutants on proteolysis of DQ-collagen IV in endothelial cells. We observed a significant decrease in proteolysis of DQ-collagen IV upon treatment with rTM EGF3-6, when compared to the control (Fig. 4.9). Conversely, endothelial cell DQ-collagen IV proteolysis significantly increased following treatment with rTM EGF3-6 V340A/D341A, compared to control and rTM EGF3-6 WT (Fig. 4.9). Additionally, treatment with rTM EGF3-6 F376A resulted in a significant decrease in DQ-collagen proteolysis when compared to the control (Fig. 4.9).

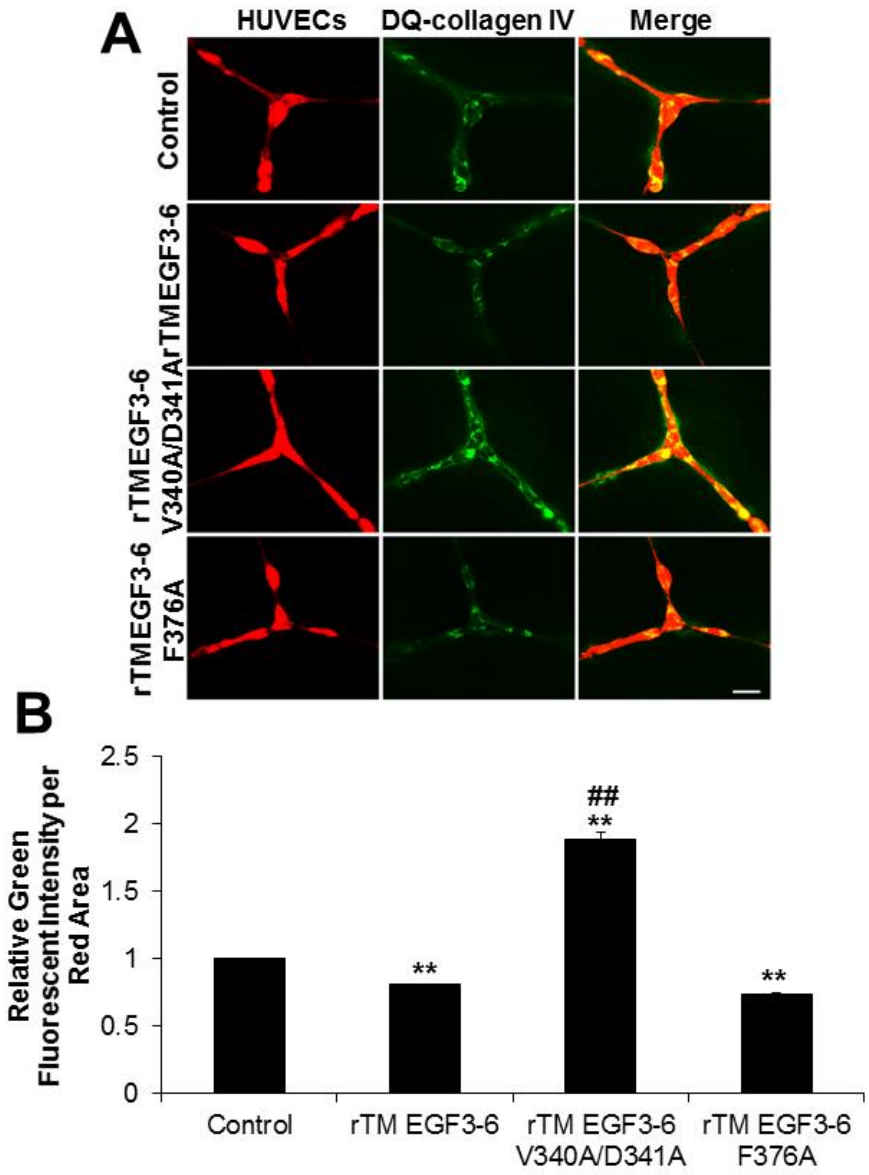


Figure 4.9: The effect of rTM EGF3-6 mutants on DQ-collagen IV proteolysis in endothelial cells.

(A) HUVECs labelled with Cell Tracker Orange (red) were seeded on BME containing 25 µg/mL DQ-collagen IV (green) and treated with 10 nM of the rTM EGF3-6 mutants. Cells were incubated for 18 hours at 37°C and then imaged using confocal microscopy, at 600× magnification. Scale bar, 30 µm. **(B)** DQ-collagen IV fluorescence was quantified and normalized to the area of CellTracker Orange fluorescence. The data are represented as the green fluorescent intensity per area of the red fluorescence, averaged over three slices of the z-stacks and expressed relative to untreated cells (Control). The data are represented as mean ± SEM from 5 independent experiments. **$p < 0.01$ versus control, ## $p < 0.01$ versus rTM EGF3-6.

Next we assessed the effect of the TM mutants on proteolysis in co-culture systems containing endothelial cells and either SUM149 or MDA-MB-231 cells. Addition of rTM EGF3-6 did not effect DQ-collagen IV proteolysis the in co-culture system, using either MDA-MB-231 (Fig. 4.10) and SUM149 (Fig. 4.11) cells. We observed a significant increase in DQ-collagen IV proteolysis in both co-culture systems when treated with rTM EGF3-6 V340A/D341A (Fig. 4.10, 4.11). We also observed an non-significant decrease in DQ-collagen IV proteolysis upon treatment with rTM EGF3-6 F376A in the HUVECs-MDA-MB-231 co-culture system, when compared to control and rTM EGF3-6 WT (Fig. 4.10). Furthermore, we observed a significant decrease in DQ-collagen IV proteolysis upon treatment with rTM EGF3-6 F376A in the HUVECs-SUM149 co-culture system (Fig. 4.11).

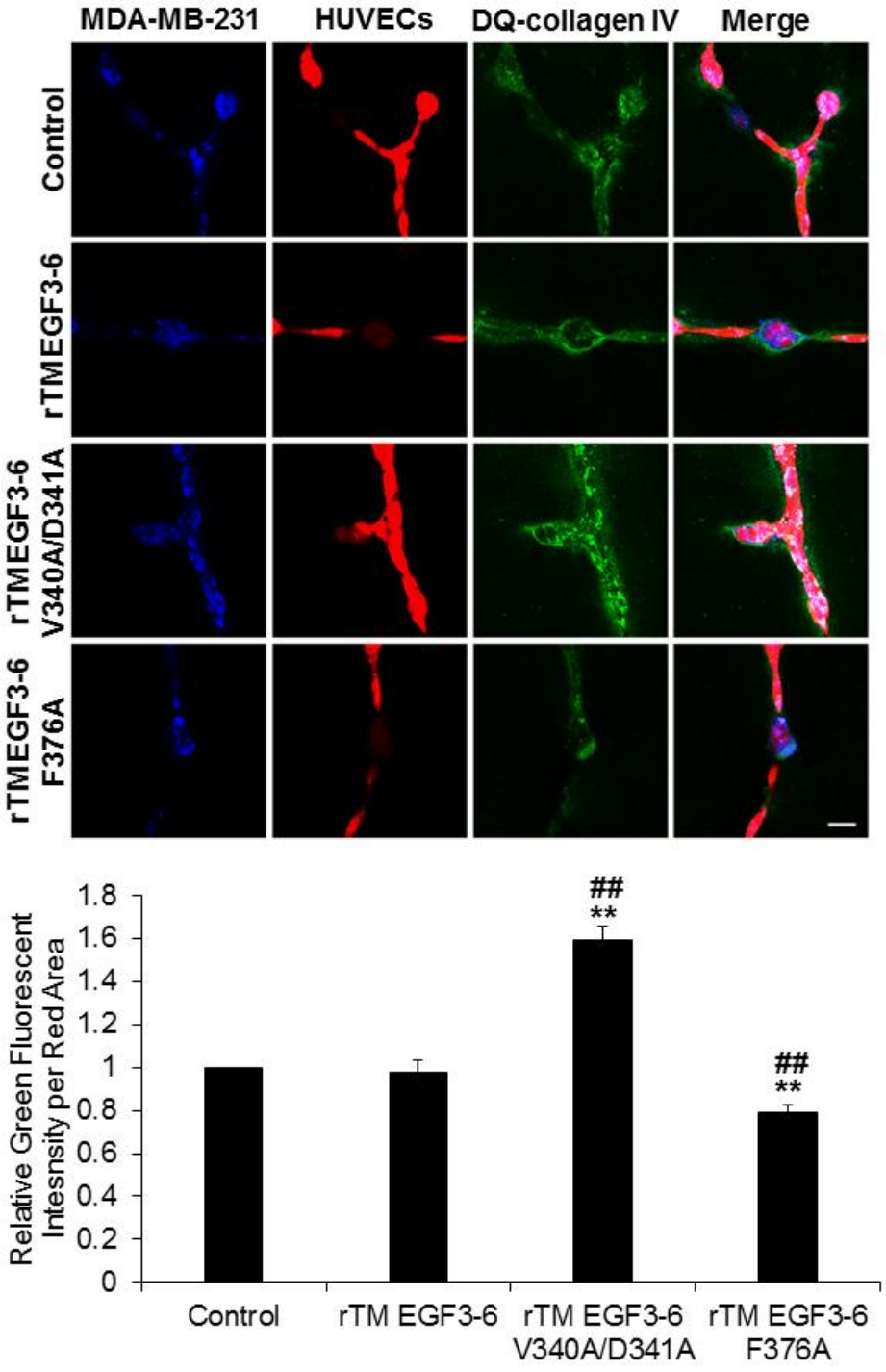

MDA-MB-231
HUVECs
DQ-collagen IV
Merge
Control
rTMEGF3-6
rTMEGF3-6 V340A/D341A
rTMEGF3-6 F376A
Relative Green Fluorescent Intesnsity per Red Area
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
##
**
##
**
Control
rTM EGF3-6
rTM EGF3-6 V340A/D341A
rTM EGF3-6 F376A

Figure 4.10: The effect of of rTM EGF3-6 mutants on DQ-collagen IV proteolysis in a co-culture system with HUVECs and MDA-MB-231 cells.
(A) MDA-MB-231 cells, labeled with Cell Trace Far Red (blue), were seeded on BME containing 25 µg/mL DQ-collagen IV (green) and incubated for 24 hours. Following the 24-hour incubation HUVECs labeled with CellTracker Orange (red) were on top of the MDA-MB-231 cells. Cells were treated with 10 nM of each of the rTM EGF3-6 variants and incubated for 18 hours prior to confocal microscopy. Images were obtained at 600× magnification. Scale bar, 30 µm. **(B)** DQ-collagen IV fluorescence was quantified and normalized to the combined area of CellTracker Orange and Cell Tracer Far Red fluorescence. The data are represented as the green fluorescent intensity per combined area of the blue and red fluorescence, averaged three slides of the z-stacks and expressed relative to untreated cells (Control). The data are represented as mean ± SEM from 6 independent experiments. **p< 0.01 versus control, ## p< 0.01 versus rTM EGF3-6.

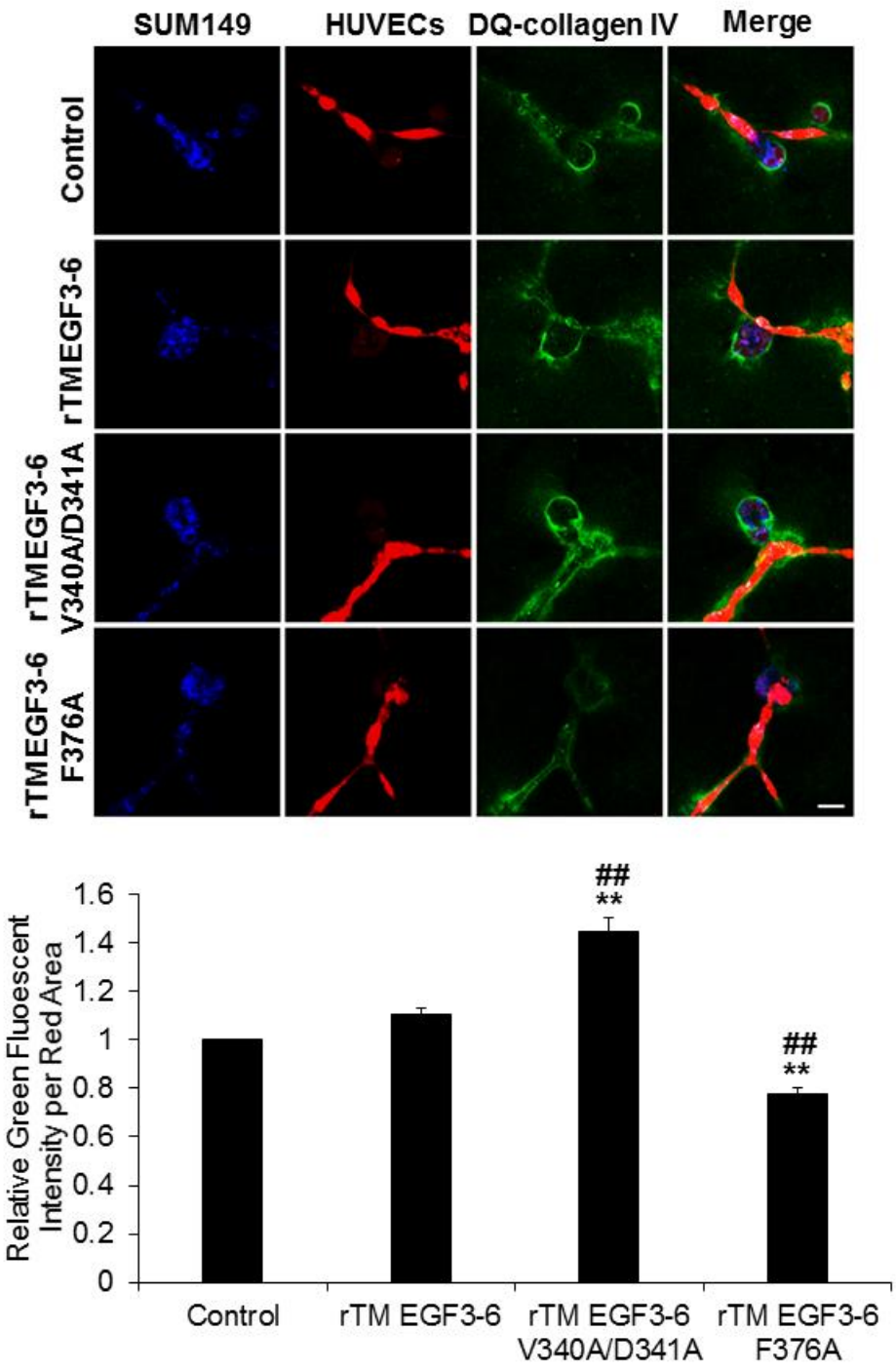

SUM149
HUVECs
DQ-collagen IV
Merge
Control
rTMEGF3-6
rTMEGF3-6 V340A/D341A
rTMEGF3-6 F376A
Relative Green Fluoescent Intensity per Red Area
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
##
**
##
**
Control
rTM EGF3-6
rTM EGF3-6 V340A/D341A
rTM EGF3-6 F376A

Figure 4.11: The effect of rTM EGF3-6 mutants on DQ-collagen IV proteolysis in a co-culture system with HUVECs and SUM149 cells.
(A) SUM149 cells, labeled with Cell Trace Far Red (blue), were seeded on BME containing 25 µg/mL DQ-collagen IV (green) and incubated for 24 hours. Following the 24-hour incubation HUVECs labeled with CellTracker Orange (red) were on top of the SUM149 cells. Cells were treated with 10 nM of each of the rTM EGF3-6 variants and incubated for 18 hours prior to confocal microscopy. Images were obtained at 600× magnification. Scale bar, 30 µm. **(B)** DQ-collagen IV fluorescence was quantified and normalized to the combined area of CellTracker Orange and Cell Tracer Far Red fluorescence. The data are represented as the green fluorescent intensity per combined area of the blue and red fluorescence, averaged three slices of the z-stacks and expressed relative to untreated cells (Control). The data are represented as mean ± SEM from 3-7 independent experiments. **p< 0.01 versus control, ## p< 0.01 versus rTM EGF3-6.

4.5 Discussion

Several groups have identified TM as an anti-metastatic factor in various cancer types. Horowitz and coworkers attributed the anti-metastatic effect of TM to its thrombin binding domain. Given that this domain is responsible for the activation of TAFI and PC, we sought to determine which substrates is important for the anti-metastatic effects of TM. Studies have demonstrated that the minimum unit of TM necessary for activation of TAFI is the c-loop of EGF-3 to EGF-6, while PC requires the interdomain loop connecting EGF-3 to EGF-4 plus EGF4-6[30]. We generated mutants of TM that differ in their ability to support TAFI and PC activation in the context of rTM EGF3-6, based on previously published mutations [30].

We measured metastatic potential of breast cancer cells based on two pro-metastatic behaviours, cell invasion and collagen degradation. While we had expected that rTM EGF3-6 would decrease these pro-metastatic behaviours, we observed an increase in both cell invasion and DQ-collagen IV proteolysis, suggesting that this variant of TM may be acting as a pro-metastatic factor. We also observed an increase in cell invasion and DQ-collagen IV proteolysis upon treatment with rTM EGF3-6 V340A/D341A. These results suggest that PC activation may promote metastatic behaviours. On the other hand, the rTM EGF3-6 F376A variant, which supports TAFI but not PC activation, did not show increases in these pro-metastatic behaviours, and in some cases decreased them to a level below that of the control. It could be that loss of ability to support PC activation abolishes the pro-metastatic effects observed with rTM EGF3-6 V340A/D341A. These results correlate with a previous study that reported an increase in breast cancer cell invasion and migration upon treatment with APC [25]. However, these also results correlate with our previous study in

which we demonstrated that addition of TAFIa decreased pro-metastatic behaviours, such as cell invasion, migration and collagen proteolysis [22]. Taken together these results suggest that TAFIa and APC may have opposing roles in cancer metastasis, where TAFIa inhibits metastatic behaviours and APC promotes cancer metastasis.

We measured angiogenic potential of endothelial cells based on tube formation and collagen proteolysis. Treatment with rTM EGF3-6 WT did not effect tube formation in any of the systems used. Treatment with rTM EGF3-6 WT significantly decreased DQ-collagen IV proteolysis in endothelial cells alone, but did not effect DQ-collagen proteolysis in the co-culture systems with endothelial cells and breast cancer cells. This contradicts a study that found use of a TM variant that included EGF1-6 and the serine/threonine domain increased endothelial tube formation [21]. Our study used a variant that lacks EGF1-2 and the serine/threonine domain, indicating a potential role for these domains in promoting angiogenesis. When we assessed cofactor ability of TM, we found that treatment with rTM EGF3-6 V340A/D341A (mutant that does not support TAFI activation) increased both endothelial tube formation and DQ-collagen IV proteolysis in all system that were tested. This indicates that manipulating the cofactor ability of TM to support PC activation may promote pro-angiogenic behaviours. Specifically, these results suggest that increasing activation of PC and decreasing activation of TAFI promotes pro-angiogenic behaviours. This is in keeping with a studies that have shown that APC can increase angiogenesis [27, 28]. Treatment with rTM EGF3-6 F376A decreased endothelial tube formation and DQ-collagen IV proteolysis in all cultured systems tested, when compared to rTM EGF3-6 WT. In general, these results suggest that stimulating of TAFI activation inhibits DQ-collagen IV proteolysis, while promoting PC activation enhances DQ-collagen IV. However, in

some cases, either rTM EGF3-6 WT had no effect, or that the effect of rTM EGF3-6 F376A was not different from control, which suggests that that in some cases TAFI activation is not playing a role, but instead the observed effect is from lack of PC activation. This is in keeping with cell invasion and tube formation results which suggest that TAFI may act as an anti-metastatic and anti-angiogenic factor, while PC is a pro-metastatic and pro-angiogenic factor. However, these results are still consistent with the idea that TAFI activation by thrombin/TM is an anti-metastatic and anti-angiogenic pathway. This is in keeping with our unpublished data that show that TAFIa is able to inhibit pro-angiogenic effects of endothelial cells. These results also suggest that, although rTM EGF3-6 WT did not effect angiogenic potential, it may be acting to both promote and inhibit angiogenesis and therefore these effects counteract each other. Therefore, TAFI and PC may have opposing roles in angiogenesis, where TAFIa acts as an anti-angiogenic factor and APC acts as a pro-angiogenic factor.

It is not surprising that TAFI and PC may have opposing roles in angiogenesis and metastasis. This is similar to their opposing functions in blood coagulation. TAFI is an anti-fibrinolytic factor, attenuating blood clot breakdown [7]. APC on the other hand is an anti-coagulant factor, inhibiting factors Va and VIIIa of the coagulation cascade, which attenuates thrombin generation [31]. Given their opposing roles and the fact that they are substrates of the same complex, the ability to modulate activation of TAFI and PC, by the thrombin/TM may be a method regulate angiogenic and metastatic behaviours. Specifically, enhancing thrombin/TM-mediated activation of TAFI, while decreasing thrombin/TM-mediated PC activation may inhibit metastatic and angiogenic behaviours.

Concentration of TM is an important regulator of TAFI and PC activation. It has been demonstrated that TM concentrations dictate which substrate would be favour for activation by the thrombin/TM complex [32]. TAFI activation is favoured at lower concentrations of TM, while PC is favoured at higher concentrations. While TM is an known anti-metastatic factor, the rTM EGF3-6 did not exhibit anti-metastatic potential, as expected. This may be due to the concentration used for these experiments. The concentration used is likely to favour activation of PC over activation of TAFI. Hence, if PC is acting as a pro-metastatic and pro-angiogenic factor, increased activation of PC may counteract the anti-metastatic and anti-angiogenic functions of TAFI. This hypothesis is supported by the fact that treatment with the mutant that only supports TAFI activation inhibits metastatic and angiogenic behaviours when compared to rTM EGF3-6 WT. This is also supported by a previous study that reported that TM increases endothelial tube length [33]. This study used much higher concentrations of TM then the current study, possibility suggesting preferential activation of PC. This suggests that promoting activation of TAFI over PC by decreasing rTM EGF3-6 WT concentrations may result in an observed anti-metastatic and anti-angiogenic response. It also cannot be overlooked that APC decreases thrombin generation which in turn decreases TAFI activation. Therefore, results observed with rTM EGF3-6 WT and rTM EGF3-6 V340A/D341A could be a combined effect of increased APC and decreased TAFIa.

Thrombin has also been implicated in cancer metastasis and tumour angiogenesis. It could be that TM acts as a thrombin sink in the tumour microenvironment, inhibiting the pro-metastatic and pro-angiogenic effects of thrombin. This is similar to the role of TM in coagulation, in which binding of thrombin to TM decreases the affinity of thrombin from

fibrinogen to PC and TAFI. Importantly, all variants of TM used in this study are able to bind to thrombin, despite having differential effects on pro-metastatic and pro-angiogenic behaviours, suggesting that this mechanism is not predominant in this model system.

Together these results indicate that manipulating cofactor ability of TM impacts both metastatic and angiogenic behaviours of breast cancer and endothelial cells, respectively. Promoting activation of TAFI over PC, using variants of TM that are specifically able to activate TAFI, may be a beneficial therapeutic approach to target both cancer metastasis and tumour angiogenesis.

4.6 References

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Chapter 5

General Discussion

Cancer metastasis is responsible for the majority of cancer-related deaths. The initial stages of cancer metastatic are facilitated in part by proteolytic events in the tumour microenvironment. Specifically, degradation of the ECM and cancer cell invasion, enables cancer cells to enter the surrounding stroma and ultimately metastasize. Proteolytic events are also important for the process of tumour angiogenesis, which also promotes cancer metastasis. In the current study we have demonstrated that TAFIa acts as an anti-metastatic and anti-angiogenic factor, inhibiting both metastatic and angiogenic behaviours in breast cancer and endothelial cells, respectively. We have demonstrated that TAFIa mediates its anti-metastatic and anti-angiogenic functions through inhibition of plasminogen activation.

5.1 Sources of TAFI in the tumour microenvironment

TAFI is synthesized predominantly in the liver, however *CPB2* mRNA and TAFI protein have been detected in other cell types, including macrophages [1]. In our work, we have detected *CPB2* mRNA in several breast cancer cell lines, with differing mRNA abundance (Fig. 2.1). Previous studies have demonstrated that *CPB2* mRNA is regulated through several mechanisms [2-4]. For example, the mRNA binding protein tristetraprolin (TTP) plays an important role in mediating the stability of *CPB2* mRNA [3] by binding to a specific *cis*-acting element in the 3'-unntranslated region of the transcript and promoting its degradation. Al-Souhibani and coworkers reported that TTP levels decreased in more invasive breast cancer cell lines, such as MDA-MB-231 [5]. We have shown that *CPB2* mRNA levels are higher in MDA-MB-231 cells (Fig. 2.1) which may be attributable to the decrease in TTP in this cell line.

We were unable to detect TAFI protein in conditioned media of any of the breast cancer cell lines tested, although this may be due to lack of assay sensitivity [6]. It is unlikely that the breast cancer cells themselves provide the major source of TAFI in the tumour microenvironment, given the low mRNA abundance in these cell lines. It is more likely that the major source of TAFI is blood plasma, with some contributions from stromal cells in the tumour microenvironment. There is an increased vascular permeability in tumour blood vessels, which results in leakage of plasma proteins from the blood vessels into the tumour microenvironment [7]. Therefore, plasma TAFI, synthesized by the liver, is able to enters the tumour microenvironment from leaky blood vessels. Stromal cells may provide another source of TAFI in the tumour microenvironment. Previous results from our laboratory have shown that macrophages express and secrete TAFI [1]. Macrophages may also be involved in modulating localized TAFI expression during inflammation, which is associated with the development and progression of cancer. Tumour-associated macrophages (TAMs) express both pro-inflammatory and anti-inflammatory mediators, both of which have been shown to increase *CPB2* mRNA and TAFI protein levels in macrophages [4, 8]. It is therefore possible that TAMs may have increased TAFI levels, though autocrine regulation, when compared to macrophages in the absence of stimulus. Other stromal cells may contribute to TAFI levels in the breast cancer microenvironment, including endothelial cell and adipocytes [1, 9]. However, while both cell types have been found to express *CPB2* mRNA, TAFI protein has not been detected in either cell type. Genetic and phenotypic changes to the stromal surroundings is a characteristic of cancer progression [10]. It is therefore possible that these changes can alter the expression of *CPB*2 in stromal endothelial cells and adipocytes. Further studies will be needed to

determine the extent of stromal contribution TAFI levels in the tumour microenvironment and whether the plasma is in fact the major source of TAFI the breast cancer microenvironment.

Results from our mRNA expression studies in Chapter 2 demonstrate that *CPB2* expression did not show any relationship with malignancy in breast cancer cells. Several clinical studies have assessed plasma concentrations of TAFI and these studies have generally found an increase in patient samples [11-14]. While these studies did not assess relationships between TAFI concentrations and metastatic disease, it could be argued that increases in plasma TAFI concentrations in patient samples contradicts the idea of TAFI as an anti-metastatic factor. However, it is necessary to point out that while TAFI concentrations may be higher in plasma, it is unknown how this influences the tumour microenvironment. We have demonstrated that *THBD* expression was generally inversely correlated to malignancy in the breast cancer cells examined, and in fact this phenomenon has been observed in several cancer types [15-17]. Therefore, it is likely that the key determinant of TAFIa action is not local concentrations of TAFI, but rather the TAFIa-generating capacity of the tumour environment, namely TM concentration, which has been shown to be inversely correlated to metastatic disease.

5.2 Activation of TAFI in the tumour microenvironment

TAFI is activated to TAFIa by thrombin, plasmin or thrombin in complex TM [18-20]. Thrombin and plasmin are both weak activators of TAFI. Thrombin-mediated activation of TAFI is dramatically accelerated when thrombin is in complex with TM [18],

leading to suggestions that the thrombin/TM complex is the physiological activator of TAFI. While we have observed decreased expression of TM in more invasive breast cancer cell lines, these levels may be sufficient to support TAFI activation. This is evident based on results we have obtained with PTCI, in (Fig. 2.3, 2.4, 2.6). We have observed pro-metastatic effects in SUM149 and MDA-MB-231 cells upon treatment with PTCI, as a result of inhibition of TAFIa. This indicates the ability of these cells to support TAFI activation, given the specificity of PTCI in this context. It is likely, however, that TAFI activation is saturated these breast cancer cells. This is evident by lack of effect in cell invasion, migration and proteolysis upon the addition of non-activated TAFI in (Fig. 2.3, 2.4, 2.6). It is expected that if these breast cancer cells activated the exogenous TAFI, there would be a decrease in pro-metastatic behaviours, as observed upon the addition of TAFIa. It is also evident that HUVECs support TAFI activation. We observed an increase in pro-angiogenic behaviours upon treatment with PTCI (Fig. 3.1, 3.4, 3.5, 3.6). This indicates that HUVECs are able to activate TAFI and inhibition of TAFIa promotes angiogenic potential (Fig. 3.1, 3.4, 3.5, 3.6). This is consistent with previous studies that have demonstrated that HUVECs support TAFI activation [21, 22]. Furthermore, unlike in breast cancer cells where non-activated TAFI did not have an effect on metastatic behaviours (Fig. 2.3, 2.4, 2.6), in endothelial cells non-activated TAFI did in fact inhibit angiogenic potential (Fig. 3.1, 3.4, 3.5, 3.6). This indicates that TAFI activation is not saturated in this cell type and the cells are able to activate exogenous TAFI. The differences observed in HUVECs compared to the breast cancer cell lines can be attributed to the levels of TM on the cell surface. It is therefore evident that TM concentrations play a role in mediating TAFI activation on the cell surface.

In patients, TM levels are inversely correlated to clinical stage and therefore thrombin/TM mediated TAFI activation in cancer would be in part dependent on expression of TM in the cancer cell [15, 16, 23]. An abolishment of TM expression would likely prevent thrombin-mediated activation of TAFI on the surface of cancer cells. However, contributions from the stromal cells in the tumour microenvironment must be considered. The influence of the tumour microenvironment on TM expression in stromal cells has not been assessed. TM is expressed in endothelial cells and macrophages [24, 25]. Inflammatory mediators have been shown to increase TM expression in macrophages, and decrease expression in endothelial cells [26]. This suggests that chronic inflammation in the tumour microenvironment may down-regulate TM in endothelial stromal cells while upregulating TM in TAMs. Decreased thrombomodulin expression in endothelial cells may results in decreased TAFI activation, however an increase in TM expression in TAMs may promote TAFI activation on these cells. Therefore, additional studies should be aimed at delineating the contributions of cancer cells and stromal cells in regulating thrombin/TM-mediated activation of TAFI in the tumour microenvironment.

In Chapter 4 we examined the role of TM in mediating pro-metastatic and pro-angiogenic behaviours. Several studies have illustrated a potential anti-metastatic role for TM. Our results demonstrated the complexity of this pathway. Unexpectedly, our soluble variant of TM (rTM EGF3-6) exhibited neither anti-metastatic potential nor anti-angiogenic potential, with the exception of DQ-collagen proteolysis of HUVEC monocultures. While both TAFI and protein C were able to be effectively activated in solution by this variant, it is unknown whether one of these substrates would be favoured upon treatment of breast cancer cells. Our results demonstrate stimulating protein C

activation, using a variant of TM that does not support TAFI activation (rTM EGF3-6 V340A/D341A), increases pro-metastatic and pro-angiogenic behaviours. This points to a role for APC in promoting pro-metastatic and pro-angiogenic behaviours. Importantly, this variant of TM, by facilitating protein C activation, inhibits thrombin formation through the intrinsic pathway of coagulation, which ultimately inhibits TAFI activation. The variant of TM that does not support protein C activation (rTM EGF3-6 F376A) demonstrated anti-angiogenic behaviour, but overall failed to inhibit pro-metastatic behaviour. Generally, this variant exhibited similar results to rTM EGF3-6 WT, indicating effects are likely due to inhibition of protein C activation. However, activity assays demonstrated that this variant was able to slightly activate protein C and it is not known what effect this would have on activation on the cell surface. Therefore, it is necessary to generate an additional variant that better inhibits protein C activation, while retaining its ability to activate TAFI. Additionally, a necessary control for these experiments should involve generation of a variant of TM that does not bind to thrombin, namely rTM EGF3-6 Q387P [27]. It would also be important to directly measure the extent of activation of protein C and TAFI, both in our *in vitro* experiments as well as in the the tumour microenvironment *in vivo*. Overall, the underlying mechanisms that facilitate thrombin/TM mediated activation of TAFI and protein C require further examination. Understanding the intricate mechanism of regulation may give insight into potential therapeutic options to promote activation of TAFI.

Binette and colleagues identified the thrombin/TM complex as the predominant activator of TAFI in a baboon model of sepsis [28]. However, it is likely that physiological activation of TAFI is dependent on the context. Plasmin-mediated activation of TAFI is accelerated in the presence of glycosaminoglycans [29]. ECM components, such as

heparan sulfate proteoglycan, are frequently overexpressed in cancers [30]. Abundance of heparan sulfate in cancer may provide an additional mechanism for TAFI activation in the tumour microenvironment. Further investigation is necessary to delineate mechanisms for TAFI activation *in vivo*, specifically in the tumour microenvironment.

5.3 Influence of TAFIa Stability

TAFIa is intrinsically unstable, possessing a half-life of approximately 8-15 minutes at 37°C [31]. With no physiological inhibitors, the spontaneous conformational change is thought to the physiological method of down-regulation of TAFIa *in vivo*. We assessed the effect of TAFIa-WT and TAFIa-CIIYQ on pro-metastatic behaviours of breast cancer cells. While TAFIa-WT was unable to inhibit pro-metastatic behaviours in breast cancer cells, we did observe a significant decrease in pro-metastatic behaviours upon treatment with TAFIa-CIIYQ (Fig. 2.3, 2.4, 2.6). These results indicate that sustained TAFIa activity over the time course of the experiment is necessary to TAFIa to effectively inhibit metastatic behaviours. Therefore, increased stability of TAFIa *in vivo* may potentially influence its anti-metastatic function. This is important as an SNP within the population, yields a variant of TAFIa (Ile325) with increased stability. In keeping with this idea, we did examine the effect of the Ile325 variant on pro-metastatic behaviours, however, like with TAFIa-WT, we did not observe an anti-metastatic effect. Our results suggest that prolonged presence of TAFIa in breast cancer inhibits metastatic potential, and therefore increasing activation or stability of TAFIa may promote its anti-metastatic behaviour.

By contrast, the dramatic differences in stability between TAFIa-WT and TAFIa-CIIYQ did not alter their effects on angiogenic potential in HUVECs. Wild-type TAFIa was equally able to significantly decrease pro-angiogenic behaviours, such as cell invasion, migration, tube formation and DQ-collagen IV proteolysis (Fig. 3.1, 3.4, 3.5, 3.6). This could be due to the presence of heparin in the HUVEC media, which increases the stability of TAFIa [29]. Importantly, the endothelium is rich in proteoglycans. Specifically, endothelial cells express glycoproteins and proteoglycans which covered the luminal surface of the cells, to form a structure called the glycocalyx [32]. Therefore, the large presence of proteoglycans on the endothelial cell surface may contribute to stabilization of TAFIa. It is also possible that the effects of TAFIa early in the time course ultimately affect the outcome of the experiment, which may not be the case for the analogous experiments using the breast cancer cell lines. Further experiments will be needed to assess the contribution of glycosaminoglycans to the stability of TAFIa in endothelial cells and breast cancer cells. Use of a variant of TAFIa with low or no affinity to heparin will give insight into whether stabilization of TAFIa by heparin does in fact enhance anti-metastatic and anti-angiogenic functions of TAFIa.

5.4 Regulation of Plasminogen Activation by TAFIa

The importance of the plasminogen activation system in cancer progression has been well-documented. The metastatic cell line MDA-MB-231 has been shown to bind and activate plasminogen more readily than the non-metastatic MCF-7 cell line [33]. This is due to an increased abundance of plasminogen receptors on the surface of these cells. Endothelial cells have also been identified to have a high capacity for plasminogen binding

on the cell surface [34]. TAFIa functions as an inhibitor of plasminogen activation, through cleavage of carboxyl-terminal lysine residues on fibrin and plasminogen receptors [35, 36]. We have directly demonstrated that TAFIa is able to attenuate plasminogen activation on the surface of breast cancer cell lines, SUM149 and MDA-MB-231, and on HUVECs (Fig. 2.2, 3.7). However, we do not know if plasmin activity itself was altered during our *in vitro* experiments. This is a difficult question to address as quantities of active enzyme are usually maintained at very low levels in biological systems due to the excess of zymogen and of specific inhibitors. Hence overcoming limits of detection using substrate assays is a significant technical hurdle. Indeed, in our zymograms for MMPs, we were not able to detect any active (cleaved) MMPs (Fig 3.8), while the DQ-collagen cleavage assays clearly showed the presence of collagenase activity in this milieu (Fig. 2.6, 3.5, 3.6).

Binding of Glu-plasminogen to plasminogen receptors gives plasminogen a more open conformation, making it more available to plasmin for cleavage into Lys-plasminogen [37-39]. This suggests that, by inhibiting plasminogen binding to plasminogen receptors, TAFIa inhibits conversion of Glu-plasminogen to Lys-plasminogen. An important characteristic of many cancers is overexpression of uPAR, which is important in accelerating plasminogen activation on the cell surface [40]. Binding of pro-uPA to uPAR promotes activation of uPA by plasmin, which is important for positive feedback mechanism in plasminogen activation. Given that TAFIa inhibits plasmin formation, this suggests that TAFIa is also able to decrease uPA activation in breast cancer, which would inhibit conversion of both Glu-plasminogen and Lys-plasminogen to Glu-plasmin and Lys-plasmin, respectively. Therefore, through cleavage of plasminogen receptors, TAFIa is

able to inhibit the positive feedback associated with acceleration of plasminogen activation, which attenuates plasmin formation on the cell surface.

The ability of TAFIa to inhibit uPA activation may also affect uPA-uPAR signaling. This signaling complex promotes cell motility through interaction with integrins on the cell surface [41]. Additionally, uPA-uPAR induces expression of pro-MMPs which facilitate ECM degradation [42, 43]. Evaluation of the effect of TAFIa on uPA-uPAR signaling is an important future goal.

As a basic carboxypeptidase, TAFIa targets carboxyl-terminal lysine residues on plasminogen receptors and therefore any plasminogen receptor with a carboxyl-terminal lysine residue is a potential substrate for TAFIa. α-Enolase, S100A10 and cytokeratin 8 are plasminogen receptors with carboxyl-terminal lysine residues [44]. They have been shown to be overexpressed in breast cancer tissue, and are therefore likely targets for TAFIa in breast cancer [45, 46]. Annexin II is an additional plasminogen receptor overexpressed in breast cancer [47]. Annexin II can be cleaved to expose a carboxyl-terminal lysine residue, which would result in it becoming a substrate for TAFIa [44]. Additionally, Annexin II can form a heterotetramer with S100A10, increasing its susceptibility to cleavage by TAFIa [48]. Therefore, there are several plasminogen receptors that are targets of TAFIa on breast cancer cells.

Stromal cells also express plasminogen receptors that can contribute to plasminogen activation in the tumour microenvironment. Plasminogen receptors such as Annexin II, Histone 2B and S100A10 are found on endothelial cells and have been shown to bind plasminogen and accelerate its activation [49, 50]. We have demonstrated that TAFIa is able to attenuate plasminogen activation on the surface of HUVECs (Fig. 3.7).

Macrophages have also been shown to express plasminogen receptors, including Plg-R_{KT} and Histone 2B [50, 51]. Both of these receptors possess carboxyl-terminal lysine residues and represent potential TAFIa substrates on macrophages. In the tumour microenvironment, it is expected that TAFIa cleaves carboxyl-terminal lysine residues from plasminogen receptors on breast cancer cells, endothelial cells and macrophages, thereby inhibiting plasminogen activation.

5.5 Role of TAFIa in Breast Cancer Metastasis

The metastatic cascade is a multi-step process that includes: (i) local invasion; (ii) intravasation; (iii) survival of the circulation; (iv) extravasation; and (v) colonization [52]. Tumour angiogenesis is important in facilitating cancer progression and promoting the metastatic process [53, 54]. Both tumour angiogenesis and cancer metastasis require proteolytic activity during multiple steps of these processes [55, 56].

Local invasion requires proteolytic events that degrade the ECM and basement membrane, facilitating cell invasion and migration into the surrounding stroma [57]. In Chapter 2, we examined the effect of TAFIa on pro-metastatic behaviours, namely local invasion, in breast cancer cells. We demonstrated that TAFIa is able to inhibit DQ-collagen IV proteolysis, cell invasion and cell migration in MDA-MB-231 cells, by attenuating plasminogen activation. We have used DQ-collagen IV proteolysis as a measure of ECM degradation. In addition to collagen IV, plasmin and MMPs have been shown to cleave other basement membrane and ECM components, including laminin, fibronectin and vitronectin. This suggests that the inhibitory effects of TAFIa on plasmin formation prevent cleavage of other ECM components that are targets of plasmin and MMPs. Therefore, it

would be advantageous to assess the effect of TAFIa on other components of the basement membrane. Importantly, in the DQ-collagen proteolysis assay cells form three-dimensional (3D) spheroids because they are grown on BME. Therefore, this assay better mimics *in vivo* conditions, since cells form 3D structures and are morphologically distinct from cells grown in a monolayer [58]. Gene expression profiles differ in cell lines grown in 3D culture compared to 2D cultures. In general, cells grown in 3D have increased expression of genes involved in cell invasion, migration and angiogenesis [59]. Given that use of 3D cultures are more representative of *in vivo* conditions, the ability of TAFIa to inhibit DQ-collagen IV proteolysis in 3D culture indicates that TAFIa can mediate proteolytic events in an environment that better mimics *in vivo* conditions.

TAFIa may also inhibit local invasion and metastasis by targeting EMT. EMT is an important phenomenon, in which cells undergo genotypic and phenotypic changes, which facilitates cell motility [60]. This process is regulated by several mediators including TGF-β1, which is known to be activated by plasmin [61]. Therefore, attenuation of plasmin formation by TAFIa may inhibit EMT by preventing TGF-β1 activation. Further studies will be needed to assess the direct effect of TAFIa on EMT, by specifically assessing whether TAFIa can inhibit TGF-β1-mediated EMT. This can be assessed by examining expression and localization of E-cadherin and N-cadherin, as well as actin cytoskeletal remodelling, all of which change during EMT. Furthermore, TM has been shown to inhibit EMT and therefore future experiment could focus on whether thrombin/TM-mediated activation of TAFI affects TM [62]. Use of mutants of TM, that support or do not support TAFIa activation, as in Chapter 3, will give insight into whether increased activation of TAFI can hinder EMT. Together, the ability of TAFIa to inhibit DQ-collagen IV

degradation, cell invasion and cell migration of MDA-MB-231 cells, suggests a role for TAFIa in mediating initial stages of the metastatic cascade, namely local invasion.

Results obtained in Chapter 3 also point to roles of TAFIa in cancer metastasis beyond local invasion. Inhibition of TAFIa, using PTCI, resulted in an increase in pro-MMP-2 and pro-MMP-9 abundance in breast cancer cells (Fig. 3.8). This points to a role for TAFIa in mediating pro-MMP-2 and pro-MMP-9 secretion, however our studies assessed the effect of TAFIa inhibition. Therefore, future experiments should assess the direct effect of TAFIa on secretion of these proteins. It would be beneficial to determine extent of activation using a more sensitive activity assay. Additionally, we should assess the mechanisms influencing TAFIa-mediated inhibition of pro-MMP-2 and pro-MMP-9 secretion. The mechanism could involve uPA-uPAR signalling, as interaction of uPA-uPAR with $\alpha_5\beta_1$ has been shown to induce pro-MMP-1 and pro-MMP-9 expression in lung cancer, by signalling through the ERK pathway [42, 43]. It would therefore be important to also assess the effect of TAFIa on mRNA abundance to determine if TAFIa can effectively inhibit pro-MMP expression. MMPs are important for the intravasation step during the metastatic process [63]. MMPs are responsible for cleaving cell-junctions to promote transendothelial migration and ultimately intravasation. Studies have also found that MMPs are important for the process of extravasation. Proteolytic events facilitate transendothelial migration from the blood vessel and promote degradation of the basement membrane, allowing the cell to enter the adjacent stroma [64]. Additionally, the plasminogen activation system plays an important role in intravasation, as inhibition of plasmin activity significantly hinders this process [65, 66]. Therefore, the ability of TAFIa

to inhibit plasminogen activation and mediate pro-MMP-2 and pro-MMP-9 secretion indicates potential roles for TAFIa in inhibiting cancer cell intravasation and extravasation.

Colonization is a key step within the metastatic cascade. To allow for propagation of a metastatic lesion, environment factors need to support metastatic growth. The effects of TAFIa on proteolysis may also influence cancer colonization. Kroger and coworkers found that treatment of mice with soluble uPAR resulted in scavenging for uPA, which decreased breast cancer metastasis to the lung by inhibiting plasminogen activation [67]. Furthermore, MMP inhibitors reduced lung colonization following intravenous injections of melanoma cells in mice [68]. The underlying mechanisms that are responsible for increased colonization with the plasminogen activation system and MMPs are largely unknown. It is likely that ECM degradation, facilitated by these proteases, promotes angiogenesis at the metastatic site. Additionally, ECM degradation at the metastatic site, similar to the primary site, releases sequestered growth factors, which promote cell growth and proliferation at the new organ [69]. Together, this suggests that TAFIa may reduce colonization, by inhibiting proteolytic events that promote the process.

Our *in vitro* studies are subject to the limitation that they do not consider tumour cell interactions with the stroma, with the exception of endothelial cells. The influences of the stromal cells have been documented in many studies [70]. Fibroblasts and TAMs release chemokines and cytokines that promote various aspects of cancer progression, including tumour growth and metastasis [70]. It would therefore be necessary to determine the effect of TAFIa on stromal interactions, specifically assessing role of fibroblasts and TAMs.

We have demonstrated a role for TAFIa in mediating breast cancer metastatic behaviours, however further experiments need to be done. Future experiments should be aimed at assessing the role of TAFIa in breast cancer metastasis *in vivo*. It will be necessary to examine the effect of TAFIa using animal models of metastasis. Syngeneic mouse models allow for assessment of metastasis in mice with an intact immune system [71]. This will allow usage of TAFI-deficient mice to understand the importance of the presence of TAFI in the tumour microenvironment. Additionally, human breast cancer cell lines implanted in immunocompromised mice are able to colonize bone, liver, lung and brain, and represent a model for assessing metastasis *in vivo* [71]. Zebrafish have also been used to assess metastasis *in vivo* [72]. Use of zebrafish is advantageous due to experiments being short, inexpensive and high throughput. Zebrafish embryos are transparent, which allows for easy visualization of cancer cells when labeled with a fluorescent dye and also allows for real-time detection. In addition, experiments can be carried out without a compromised immune system, as in many traditional *in vivo* cancer models. Finally, zebrafish expressed orthologues corresponding to TAFI, TM, and prothrombin and therefore represent a viable method to examine anti-metastatic potential of TAFIa.

5.6 Role of TAFIa in Modulating Tumour Angiogenesis

In Chapter 3, we examined the effects of TAFIa on pro-angiogenic behaviours in endothelial cells and in co-culture systems with endothelial cells and breast cancer cells. TAFIa has been previously shown to inhibit tube formation on a fibrin matrix, through inhibition of plasminogen activation [73]. In the current study we demonstrate the ability of TAFIa to inhibit tube formation on BME. TAFIa was able to decrease tube formation

both in monoculture and in co-culture systems with breast cancer cells. Importantly, our *in vitro* tube formation assays utilized BME to mimic *in vivo* conditions, in which endothelial cells are able to proliferate, migration and differentiate to form tube-like structures. However, cancer cells play an important role in facilitating tumour angiogenesis through paracrine signaling [74]. Secretion of pro-angiogenic factors by cancer cells, such as VEGF, promotes endothelial cell activation and subsequent angiogenesis [74]. Therefore, our co-culture experiments allowed for examination of tube formation in an environment that better mimic *in vivo* tumour angiogenesis. Endothelial cells formed tube-like networks that connected individual breast cancer spheroids grown on BME, which indicates that the breast cancer cells are able to modulate the angiogenic behaviours of the endothelial cells. However, we did not observe any differences in the effects of TAFIa in co-culture experiments when compared to monoculture experiments. It could be that the high levels of TM on the endothelial cells dominate in this system and therefore the ability of the breast cancer cells to promote TAFI activation is inferior to promotion of activation by the endothelial cells. This could be examined by knockdown of TM expression in endothelial cells to determine whether the breast cancer cells alone can promote TAFI activation to inhibit tube formation.

ECM degradation facilitates endothelial cell invasion into the surrounding stroma during angiogenesis. We observed inhibitory effects of TAFIa on VEGF-mediated cell invasion and migration, as well as DQ-collagen IV proteolysis. Endothelial cells require angiogenic stimulation, such as VEGF to promote cell invasion and migration. VEGF is a potent activator of angiogenesis, inducing endothelial cell migration and proliferation and was therefore used to induce cell invasion and migration. These results indicate a potential

role for TAFIa in mediating ECM degradation and subsequent cell invasion required during the process of angiogenesis. We also used co-culture experiments to demonstrate that TAFIa is able to inhibit DQ-collagen IV proteolysis in an environment that better mimics tumour angiogenesis. Furthermore, we observed a decrease in VEGF-mediated migration of endothelial cells, which is an important in vessel sprouting. Overall, we demonstrated that TAFIa is able to inhibit pro-angiogenic behaviours, although further studies are needed to assess whether TAFIa can exhibit similar functions *in vivo*. The Matrigel plug assay is considered one of the best methods to screen pro-angiogenic and anti-angiogenic factors [75]. Additionally, the chick choriollantoic membrane (CAM) assay is an effective method to study tumour angiogenesis, as cross-species xenografts are tolerated [76]. This would allow xenotransplantation of human breast cancer cells to assess tumour angiogenesis. Zebrafish are also used to study angiogenesis *in vivo*. Transparency of zebrafish embryos allow for convenient visualization of blood vessel formation. Use of animal models will give insight into the ability of TAFIa to exhibit anti-angiogenic functions *in vivo*.

5.7 Alternative Mechanisms of Inhibition of Metastasis by TAFIa

In addition to cleaving plasminogen receptors, TAFIa cleaves carboxyl-terminal lysine and arginine residues from inflammatory mediators, including thrombin-cleaved osteopontin, bradykinin, anaphylatoxins C3a and C5a and plasmin-cleaved chemerin. In addition to their roles in inflammation, these TAFIa substrates have also been shown to be involved in cancer.

Osteopontin levels have been correlated with an invasive phenotype in several cancer types, including breast cancer [77-80]. Studies have demonstrated that osteopontin

is functionally important in cancer progression and metastasis. Specifically, osteopontin upregulates expression of uPA, pro-MMP-2 and pro-MMP-9, increasing matrix degradation, cell invasion and cell migration [81-83]. Thrombin has been shown to cleave osteopontin, resulting in exposure of a carboxyl-terminal lysine residue. This form of osteopontin has also been found to promote cell invasion and metastasis [84]. Furthermore, overexpression of osteopontin was found to induce angiogenesis in glioma [85]. Liaw and coworkers demonstrated that osteopontin stimulates angiogenesis through binding to $\alpha_v\beta_3$ integrin [86]. Interaction of osteopontin with this integrin has been shown to induce VEGF expression, ultimately stimulating angiogenesis [87]. However, Beausoleil and coworkers observed increased metastatic lesions in mice upon deletion of the thrombin-cleavage domain of osteopontin [88]. Thrombin-cleaved osteopontin possesses a carboxyl-terminal lysine and therefore can be cleaved by TAFIa. It is possible that cleavage by TAFIa represents a mechanism of regulation of osteopontin function on the cell surface and therefore removal of the thrombin-cleavage domain would result in the inability of TAFIa to regulate the invasive and metastatic functions of osteopontin.

Bradykinin activates several signaling pathways involved in cancers, specifically MAPK, ERK, PKC and NF-κB [89-92]. Bradykinin has been shown to increase prostate cancer cell migration and expression of pro-MMP-9 [93]. Montana and coworkers reported that bradykinin also promotes cell invasion in glioma [94]. They found that bradykinin upregulates IL-6 expression, which has been previous shown to increase cancer cell invasion and metastasis [95-97]. Yu *et al.* found that bradykinin was able to stimulate VEGF expression in prostate cancer cells and also promote endothelial tube formation [98]. Together these studies suggest functions for bradykinin in cancer metastasis and

angiogenesis. Basic carboxypeptidase cleavage of bradykinin has been found to inactivate this ligand and therefore illustrates a method of regulation [99]. TAFIa has been shown to inactivate bradykinin by cleaving its carboxyl-terminal lysine residue [100]. Therefore, in addition to regulating plasminogen activation, TAFIa may regulate bradykinin in the tumour microenvironment. This would result in a decrease in bradykinin-mediated cell invasion and migration.

Studies have shown that the complement system is active in cancer cells, both *in vitro* and *in vivo* [101, 102]. Nitta and coworkers have demonstrated that C5a enhances cancer cell invasion and promotes secretion of pro-MMPs in cancer cells [103]. C5a has also been found to stimulate MMP production in macrophages [104]. Piao *et al.* reported that colon cancer cells secrete elevated levels of C5a and that blocking of C5a decreased hepatic metastases of colon cancer in mice [105]. C5a also promotes endothelial cell migration, proliferation and vessel formation, both *in vitro* and *in vivo* [106]. Both C3a and C5a have been shown to induce VEGF expression, which stimulated neovascularization [107]. Inactivation of complement factors by TAFIa may therefore decrease complement-mediated metastatic and angiogenic functions.

Future experiments should also be aimed at identifying additional mechanisms of TAFIa function in cancer metastasis. Experiments should be aimed at examining the role of TAFIa in mediating the pro-metastatic and pro-angiogenic effect of osteopontin, bradykinin and anaphylatoxin C5a. It is reasonable to expect that these non-plasminogen activation-mediated events are occurring in our *in vitro* system and may have contributed to the effects observed in the current study. It would therefore be important to discern the contribution of these mechanisms on the anti-metastatic and anti-angiogenic effects

observed by TAFIa compared to the effects-mediated by inhibition of plasminogen activation. These contributions could be examined in a system deficient of plasminogen, through depletion of plasminogen from serum. Metastatic and angiogenic assays can be carried out using plasminogen-deficient serum to assess whether TAFIa can inhibit metastatic and angiogenic behaviours independent of plasminogen activation, *in vitro*.

5.8 Therapeutic Potential of the TAFI pathway

The work in this thesis has, for the first time, identified TAFIa as a potential anti-metastatic factor in breast cancer. We have demonstrated that attenuating plasminogen activation by targeting plasminogen receptors, which may be the mechanism by which TAFIa inhibits metastatic and angiogenic behaviours. Given that plasminogen receptors are overexpressed in a number of cancers, they represent a vital therapeutic target. Plasmin formation is upstream of MMP activation and therefore targeting plasminogen activation may have more beneficial clinical outcomes than trials with MMP inhibitors.

Targeting the TAFI pathway in the breast cancer microenvironment either by promoting activation of TAFI, or by increasing TAFIa stability may represent therapeutic strategies. Promoting TAFI activation can be accomplished using a variant of TM that only supports activation of TAFI and not of protein C. It would be advantageous to use TM as a therapeutic, given that soluble TM (Solulin) is currently in clinical trials for treatment as an anti-coagulant. A phase I clinical trial indicated that Solulin is safe and tolerated with no reports of bleeding complications [108]. Use of TM as a therapeutic may also be beneficial with regards to thrombin in the tumour microenvironment. Given that thrombin

has metastatic functions, TM may act as a thrombin sink and change its substrate specificity away from its metastatic functions.

Increasing the stability of TAFIa may also be a beneficial therapeutic strategy. We have shown that increasing the stability of TAFIa resulted in inhibition of pro-metastatic behaviours in breast cancer cell lines. A stabilizing agent such as an antibody that binds to the dynamic flap, which prolongs the activity of TAFIa, may have similar inhibitory effects. Whether utilizing a TAFIa stabilizing agent or a variant of soluble TM, it would be necessary to establish localized therapies to avoid side effects related to bleeding or thrombosis. It is well known that agents introduced directly into the vascular system do preferentially accumulate at tumour sites due to the leaky nature of the tumour vasculature [63]. Alternatively, liposomes are an attractive method for providing localized therapies [109]. Use of liposomes conjugated to a selective marker enables drug delivery directly to the tumour site and may represent a method to provide localized TAFIa treatment in breast cancer patients. Taken together, we have demonstrated that TAFIa plays an inhibitory role in breast cancer metastatic and angiogenic behaviours and this pathway may represent a therapeutic strategy.

5.9 Concluding Remarks

The process of cancer metastasis relies on elaborate interactions within the tumour microenvironment. Proteolytic events mediated by cancer cells, stromal cells and the ECM promote the metastatic process. Specifically, proteases such as plasmin and MMPs, stimulate cancer progression by facilitating ECM degradation, cell invasion and ultimately

cancer metastasis. The ability to control and down-regulate these proteolytic events may represent a therapeutic strategy to inhibit the metastatic process. In this current study we have demonstrated, for the first time, a direct effect of TAFIa in inhibiting metastatic and angiogenic behaviours in breast cancer, *in vitro*. We have shown that TAFIa regulating plasminogen activation on the cell surface, *in vitro*. TAFIa is able to inhibit breast cancer and endothelial cell invasion, migration and ECM proteolysis, likely through inhibition of plasminogen activation. Our studies have also found that TAFIa can inhibit endothelial tube formation, which is an indicator of angiogenic potential. Taken together, our results have demonstrated a role for TAFIa in regulating metastatic and angiogenic processes *in vitro*. Further studies will be needed to assess the role of TAFIa on metastasis and angiogenesis *in vivo*. TAFIa may represent a novel therapeutic strategy aimed at inhibiting breast cancer metastasis and tumour angiogenesis.

5.10 References

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APPENDIX:

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2 messages

Zainab Bazzi <bazziz@uwindsor.ca> Thu, Sep 22, 2016 at 10:07 AM
To: Rocco Romagnuolo <romagnu@uwindsor.ca>

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Rocco Romagnuolo <romagnu@uwindsor.ca> Thu, Sep 22, 2016 at 10:11 AM
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Good morning,

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2007-2011 Bachelor's of Science (BSc.), Honours, Biochemistry and Biotechnology, with thesis
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PUBLICATIONS:

JOURNAL ARTICLES

1. Bazzi ZA, Balun J, Cavallo-Medved D, Porter LA, Boffa MB. Activated Thrombin-activatable Fibrinolysis Inhibitor Attenuates the Angiogenic Potential of Endothelial Cells: Potential Relevance to the Breast Tumour Microenvironment. *Clinical and Experimental Metastasis.* Submitted. CLIN-D-16-00098

2. Bazzi ZA, Lanoue DL, El-Youssef M, Tubman J, Cavallo-Medved D, Porter LA, Boffa MB. Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) attenuates breast cancer cell metastatic behaviors through inhibition of plasminogen activation and extracellular proteolysis. *BMC Cancer*. 2016 May 24;16(1):328.

3. Komnenov D, Scipione CA, Bazzi ZA, Garabon JJ, Koschinsky ML, Boffa MB. Inflammatory cytokines reduce thrombin activatable

fibrinolysis inhibitor (TAFI) expression via tristetraprolin-mediated mRNA destabilization and decreased binding of HuR. *Thrombosis & Haemostasis*. 2015 Aug;114(2):337-49.

CONFERENCE PUBLICATIONS

1. Bazzi ZA, Balun JL, Boffa, MB. Thrombin activatable fibrinolysis inhibitor is a regulator of angiogenic potential in endothelial cells [abstract]. *Arterioscler Thromb Vasc Biol.*

2. Bazzi ZA, Rudy D, Porter LA, Cavallo-Medved D, Boffa MB. Activated thrombin activatable fibrinolysis inhibitor is a novel anti-metastatic factor in breast cancer [abstract]. In: Proceedings of the Thirty-Seventh Annual CTRC-AACR San Antonio Breast Cancer Symposium: 2014 Dec 9-13; San Antonio, TX. Philadelphia (PA): AACR*; Cancer Res* 2015;75(9 Suppl):Abstract nr P1-07-32.

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2. Bazzi, ZA, Lanoue, DL, El-Youssef, M, Cavallo-Medved, D, Porter, L, Boffa, MB. Stabilization of activated thrombin activatable fibrinolysis inhibitor enhances its anti-metastatic potential in breast cancer. Canadian Cancer Research Conference, Montreal, Canada. (2015) Poster Presentation.
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5. Bazzi, ZA, Rudy, D, Cavallo-Medved, D, Porter, L, Boffa, MB. TAFI is Novel Inhibitor of Breast Cancer Cell Invasion and Migration. Windsor Cancer Research Group (WCRG) Research Conference, Windsor, Canada. (2012) Poster Presentation.

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Casino Windsor Cares/Gail Rosenblum Memorial Breast Cancer Research Scholarship	2015
University of Windsor Graduate Student Society Scholarship	2015
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