THE UNIVERSITY OF HULL

Examination of the Up-regulation and Transfer of Tissue Factor to Endothelial Cells by Tumour-Derived Microparticles

being a Thesis submitted for the degree of

MSc. Biology

in the University of Hull

by

Pui Mei, Mah

BSc (University of Hull)

March 2013

Abstract

Overexpression of tissue factor and its release as microparticles is a common feature of aggressive cancer. Furthermore, increased levels of tissue factor have been detected in the tumour associated microvascular endothelial cells. The accumulation of active tissue factor on the luminal surface of blood vessels generates a thrombogenic surface which can lead to thrombus formation. Although tissue factor has been shown to be present within the endothelial layer in cancer patients, the source of this tissue factor still remains unknown. It has been shown that tissue factor can be transferred between different types of cells through microparticles. In addition, endothelial cells have been shown to be capable of expressing tissue factor in response to microparticles. Therefore, this study aimed to investigate the source of endothelial cell-associated tissue factor by analysing the transfer of cancer-derived tissue factor by microparticles, to microvascular endothelial cells, *in vitro*. The study also evaluated the *de novo* expression of tissue factor within endothelial cells in response to these microparticles. Human dermal blood endothelial cells (HDBEC) were incubated with tissue factor-bearing, or tissue factor-deficient microparticles isolated from the medium of MDA-MB-231 or MCF-7 cell lines respectively. Subsequently, HDBEC-surface tissue factor antigen was quantified over 360 min using a tissue factor-specific ELISA. Surface tissue factor activity was determined using a chromogenic assay and the presence of phosphatidylserine on the HDBEC surface was monitored using annexin V-labelling. Two distinct surface tissue factor antigen peaks were detected at 30 and 120 min following the incubation of the cells with tissue factor-bearing microparticles but not tissue factor-deficient microparticles. However, only the latter peak at 120 min was accompanied with high tissue factor activity. This activity was attributed to the exposure of phosphatidylserine on the endothelial cell surface demonstrated by the increased annexin-V labelling at

90 min. Analysis of tissue factor mRNA, up to 24 h, did not reveal any *de novo* expression of tissue factor in response to the microparticles. In addition, pre-incubation of HDBEC with a dynamin inhibitor, Dynasore, resulted in the disappearance of the latter tissue factor antigen exposed on the cell surface at 120 min. These data suggest that tumour-derived tissue factor can be transferred to endothelial cells on exposure to tumour derived microparticles. Furthermore, tissue factor-bearing microparticles are internalised by endothelial cells and may then be recycled onto the endothelial cell surface in a highly active form. This in turn may result in a substantial increase in the procoagulant potential on the surface of endothelial cells leading to thrombosis.

Acknowledgement

I would like to thank my supervisor, Dr.Camille Ettelaie for her continued support, guidance and advice throughout my whole MSc programme. I would also like to thank Mary for her guidance, support and patience during the whole project. I would like to extend my gratitude to the “tissue factor” group namely Anna, Clover and Azza who have contributed time, knowledge and support. Last but not least, I am thankful to my parents for their love, moral and financial support throughout my studies.

List of Abbreviation

AP	Alkaline phosphate
BHK	Baby hamster kidney
BSA	Bovine serum albumin
BSAP	Barium sulphate-adsorbed plasma
cDNA	complementary Deoxyribonucleic acid
DiI	1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
*E.*coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescence protein
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FIX	Factor IX
FVII	Factor VII
FX	Factor X
FXIII	Factor XIII
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluprescence protein
HDBEC	Human dermal blood endothelial cells
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HRP	Horseradish peroxidase
LPS	Lipopolysaccharide

mRNA	messenger Ribonucleic acid
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
PAR2	Protease activated receptor 2
PAR2-AP	Protease activated receptor 2-agonist peptite
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SDS	Sodium dodecyl sulfate
SOC	Super optimal broth with catabolite repression
TBE	Tris-borate-EDTA
TEMED	N,N,N’,N’-tetramethylethylenediamine
TF	Tissue factor
Tm	Melting temperature
TMB	3,3’,5’5 –tetramethylbenzidine
TNF	Tumour necrosis factor
VTE	Venous thromboembolism

List of Figures

List of Tables

Tables of Contents

Chapter 1

Introduction

1.1 Cancer and venous thromboembolism

Venous thromboembolism (VTE) is characterized by the development of fibrin-rich clots that form on the surface of endothelium in the absence of vessel wall injury (Sevitt 1974). The link between VTE and cancer was suggested by Trousseau in 1865. VTE is recognised as the second common cause of morbidity and mortality in cancer patients (Sørenson *et al.* 2000). Statistics show that up to 90% of cancer patients develop VTE at some stage of the disease and this is often associated with poor survival rate (Rickles *et al.* 2003, Milsom *et al.* 2007). In fact, a four-fold increase in the risk of VTE is observed in cancer patients, compared to the general population and the use of chemotherapy may further exacerbate this risk up to seven-fold (Heit 2005).

A number of studies have associated the incidence of VTE to the procoagulant protein, tissue factor (Falanga 2005, White *et al.* 2007, Khorona *et al.* 2007). Tissue factor may be over-expressed by tumour cells, particularly in the more aggressive types of cancer such as brain, pancreatic and prostate cancers (Kakkar 1995, Falanga 2005) and is also detected in tumour-associated vasculature. However, there does not appear to be any direct correlation between the surface tissue factor expressed on tumour cells and incidence of VTE (Furie & Furie, 2006, Uno *et al.* 2007). Furthermore, it has been shown that tumour cells are capable of shedding tissue factor as cell-derived microparticles (Tsselaar *et al.* 2007, Zwicker 2009). In support of this, an increasing number of studies report the increased levels of microparticles in the plasma of cancer patients and particularly, in cancer patients who develop VTE (Hron *et al.* 2007, Khorona *et al.* 2008, Zwicker *et al.* 2009, Tsselaar *et al.* 2007). Therefore, it has been suggested that microparticles may be a direct link between development of VTE and cancer. Although cancer cells are shown to release tissue factor as microparticles, the underlying mechanism of the shedding of microparticles from cancer cells is yet to be

determined. Recently, the release microparticles from cancer cells has been suggested to associate with the activation of the tumour suppressor protein p53 signalling pathway, in response to stress. Consequently, the p53 signalling pathway up-regulates the expression of tumour suppressor activated pathway-6 (TSAP6) protein which facilities the secretion of microparticles (Yu *et al.* 2009).

1.2 Haemostasis and blood coagulation pathway

VTE occurs as a consequence of breakdown in the normal haemostatic control mechanism. Haemostasis is the protective physiological response to vascular injury to prevent excessive blood loss and to maintain the integrity of blood vessels. The coagulation mechanism involves two distinct, but interrelated pathways (the intrinsic and extrinsic pathways) (Figure 1.1). The intrinsic and extrinsic pathways converge at the point of activation of factor X (FX), leading to the common pathway of thrombin generation and fibrin clot formation. Tissue factor is the primary initiator of the extrinsic pathway of blood coagulation (Nemerson 1995). Due to the extravascular localisation, tissue factor has no direct contact with the blood under normal conditions. Upon disruption of the endothelial layer, the extrinsic coagulation pathway is triggered by the exposure of tissue factor and the recruitment of the zymogen coagulation proteases within the bloodstream. Tissue factor binds factor VIIa (FVIIa) to form the TF-VIIa complex. The TF-FVIIa complex in turn activates factor X (FX) to its active form, factor Xa (FXa) or tenase by proteolytic conversion. TF-FVIIa complex also activates factor IX (FIX) to (FIXa), feeding back onto the intrinsic pathway. FXa, in the common pathway, forms a prothrombinase complex with its cell-surface cofactor Va to generate thrombin from prothrombin. Thrombin in turn catalyses the conversion of fibrinogen into fibrin, resulting in the formation of a loose fibrin mesh network. Factor XIII (FXIII) stabilizes the fibrin mesh network (Rak *et al.* 2009, Camerer *et al.* 2006).

In addition to fibrin deposition, thrombin promotes platelet activation and aggregation through the activation of protease-activated receptors which leads the formation of clot that covers the damage surface of the blood vessel (Rak *et al.* 2009, Camerer *et al.* 2000).

1.3 Cell-derived microparticles

Microparticles are membrane-derived vesicles that are released from a number of cell types including platelets, leukocytes, endothelial cells and cancer cells by exocytic budding of the plasma membrane (Fevrier *et al.* 2004, Leroyer *et al.* 2010, Nomura *et al.* 2008). Although not clearly defined, microparticles have characteristics that distinguish them from exosomes. For example, microparticles have a diameter range of 0.1-1 μm (Cocucci *et al.* 2009) while exosomes are 0.03-0.1 μm in size (Conde-Vancells *et al.* 2008). Microparticles may be released from activated or apoptotic cells. In addition, microparticles contain a cytoskeleton but do not possess a nucleus. The microparticle surface is made up of a phospholipid bilayer, with the outer membrane mainly composed of negatively charged phospholipids such as phosphatidylserine (Lechner & Weltermann, 2008, Hugel *et al.* 2005, Burnier *et al.* 2009). Microparticles have heterogeneous protein composition and carry different cell-specific antigens on their membrane, which reflect the origin of the microparticles. Therefore, the source of microparticles may be determined by the distinct antigens displayed on the membrane of the microparticle (Lechner & Weltermann 2008, VanWijk *et al.* 2003).

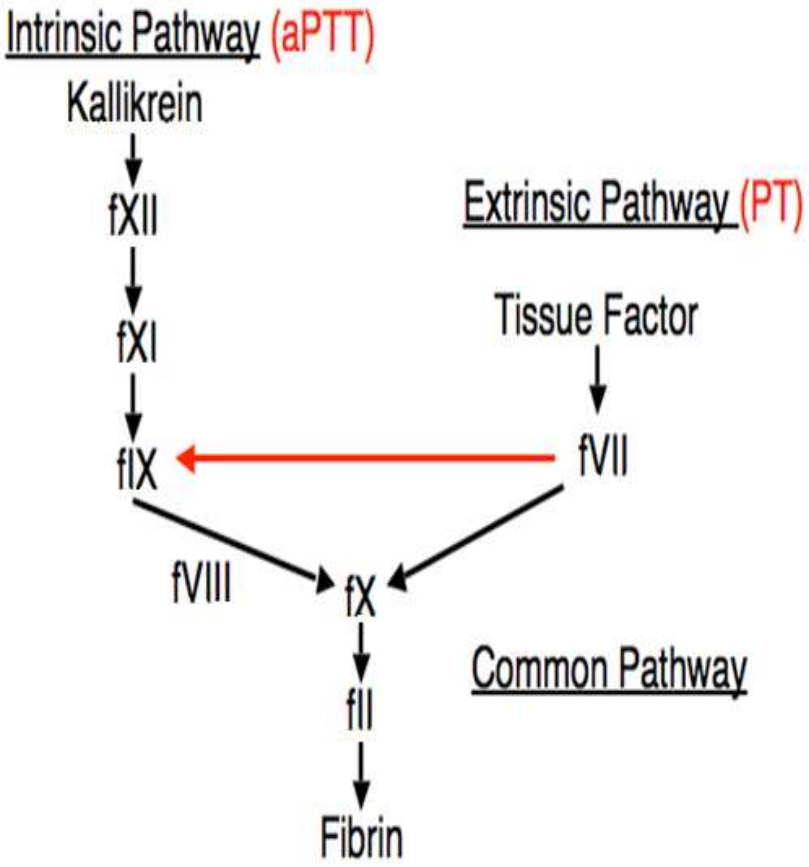

Figure 1.1: Schematic representation of the blood coagulation cascade

Upon disruption of endothelial cells, extrinsic coagulation pathway is triggered when tissue factor is exposed to the bloodstream. Tissue factor binds to FVII to form TF-FVII complex. TF-VII complex will then activate FX to FXa. In addition, the TF-FVII complex activates FIX to FIXa, feeding back on the intrinsic pathway. The intrinsic and extrinsic pathways converge at the point of factor X activation, leading to common pathway that generates fibrin clots.

For example, the presence of antigen CD14 on the microparticle surface indicates a monocytic origin while platelet-derived microparticles may be identified by the presence of glycoproteins IIb-IIIa or CD42a. Similarly, endothelial cells-derived microparticles have been shown to display surface CD31 or CD146 (Nomura *et al.* 2008, Jimenez *et al.* 2003).

Following the activation of cell or induction of apoptosis, the rise in cytosolic calcium concentration induces the cytoskeletal-dependent rearrangement of the plasma membrane. These changes in the cytoskeleton result in the blebbing of the cell surface membrane, which lead to the release of microparticles (Nomura *et al.* 2008, Martinez *et al.* 2005, Zwaal & Schriot 1997). Cellular activation or apoptosis also causes the exposure of the negatively charged phospholipids on cells (Nomura *et al.* 2008, Martinez *et al.* 2005) (Figure 1.2). The distribution of phospholipids within the cell membrane is regulated by three phospholipid transporters. These transporters include the inward-directed pump (flippase), an outward- directed pump (floppase) and scramblase. Under resting conditions, flippase is specific for the translocation of phosphatidylserine and phos-phatidylethanolamine from the outside to the inside of lipid bilayer membrane while floppase transport phosphatidylcholine and sphingomyelin from the inner to outer layer. Consequently, phospholipids are asymmetrically distributed in the cell membrane under resting conditions. Once the cell is activated, scramblases enable all phospholipids to flow down their concentration gradients. This unspecific bidirectional migration of phospholipids favours phospholipids randomization (Bevers *et al.* 1999, Hugel *et al.* 2005).

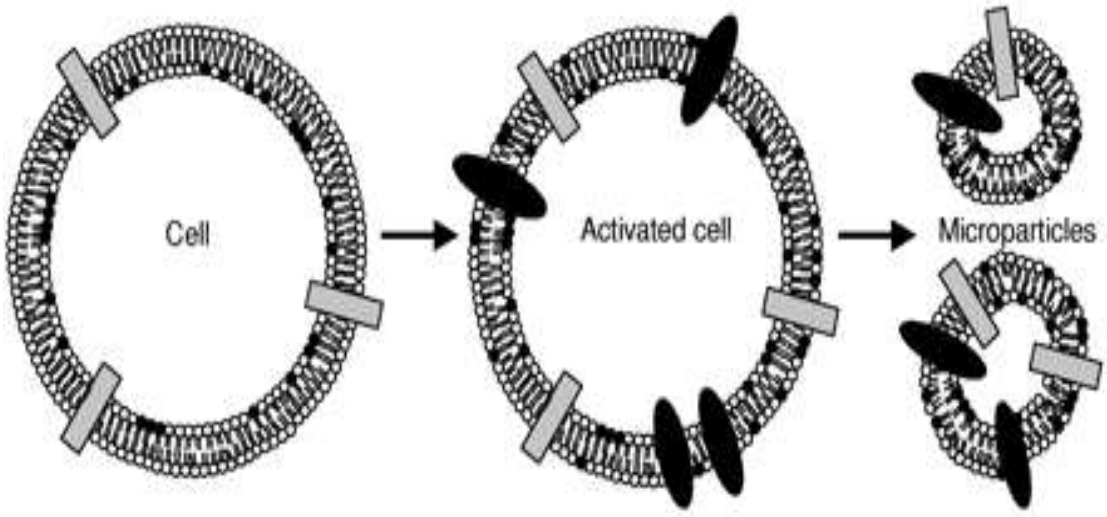

Figure 1.2: Schematic representation of microparticle generation and composition.

Negatively charged phospholipids are presented by the black dots. The grey rectangles represent cell-specific antigens, e.g., CD4 for T-helper cells. In resting cells, the negatively charged phospholipids such as phosphatidylserine, are located in the inner layer of lipid bilayer. When cell is activated, negatively charged phospholipids are relocated to the outer layer of lipid bilayer. Therefore, the release of microparticles is exposed to the negatively charged phospholipids together with the transferred of molecules that have been up-regulated during cell activation (represented by the black oval).

In addition, following calcium influx, the activity of floppase is activated while the activity of flippase is concomitantly inhibited. As a consequence, the phospholipids asymmetry between the inner and outer leaflets is disrupted (Bevers *et al.* 1999, Hugel *et al.* 2005, Burnier *et al.* 2009, Morel *et al* 2011).

1.4 The procoagulant activity of microparticles

The presence of phosphatidylserine on the surface of microparticles supports blood coagulation by providing a surface which facilitates the assembly of the tenase and prothrombinase enzyme complex (Gilbert *et al.* 1991, Nomura *et al.* 2008, Morel *et al.* 2011). In addition to the surface phosphatidylserine, microparticles also harbour tissue factor (Dignat-George & Boulanger 2011, Tushuizen *et al.* 2011). In fact, the majority of the circulating tissue factor detected within blood circulation is associated with microparticles (Tilley *et al.* 2008). Monocyte, leukocyte and endothelial derived-microparticles have all been shown to contain tissue factor. Moreover, tissue factor may be transferred between different cell types via microparticles (Rauch *et al.* 2000). For example, tissue factor has been shown to transfer from leukocyte-derived microparticles to platelets (Rauch *et al.* 2000, Del Conde *et al.* 2005). Therefore, it is possible for cells to contain surface tissue factor derived from other cell types without transcription of tissue factor DNA.

Microparticles may accumulate at the site of injury and contribute to the development of thrombus through the binding of P-selectin glycoprotein 1 (PSGL-1) to P-selectin. P-selectin is an adhesion molecule expressed on activated endothelial cells and activated platelet. PSGL-1 is found to be present on monocyte (Falati *et al.* 2003), leukocyte (Vandendries *et al.* 2004), and cancer-cell derived-microparticles (Thomas *et al.* 2009). Falati *et al.* (2003) showed that monocyte-derived microparticles are recruited to the

developing thrombus through the interaction of P-selectin. This in turn results in the accumulation of tissue factor and subsequently thrombin generation. Furthermore, Thomas *et al.* (2009) showed that by blocking P-selectin with an antibody, tumour-derived microparticles accumulation may be prevented at the site of injury. Therefore, P-selectin and PSGL-1 have been suggested to have a crucial role in the recruitment of tissue factor-bearing microparticles.

1.5 Tissue factor

Tissue factor (also known as coagulation factor III, thromboplastin or CD142) is a 47 kDa integral membrane glycoprotein (Rak *et al.* 2009, Viles-Gonzalez *et al.* 2004) encoded by a gene that contains six exons located on chromosome 1 (p21-22) (Kao *et al.* 1988). Human tissue factor consists of 295 amino acid, and the first 32 amino acid of which serve as a signal peptide that encoded from exon 1. The mature protein is composed of a 219 amino acid extracellular domain (encoded from exon 2 to 5), a 23 amino acid transmembrane domain and a 21 amino acid intracellular C-terminal tail (encoded from exon 6) (Forster *et al.* 2006, Mackman 2007) (Figure 1.3 & Figure 1.4).

Under physiological conditions, tissue factor is expressed by extravascular cells, including smooth muscle cells and adventitial fibroblast, but not expressed by blood or endothelial cells. However under pathological conditions such as injury or malignancy, endothelial cells, platelets and monocytes may express tissue factor. Tissue factor is expressed in a tissue-specific pattern with high levels detected in brain, lung, heart, kidney and placenta (Mackman *et al.* 2004, Chu 2011), providing haemostatic protection for these vital organs.

In addition its role as a regulator of haemostasis, tissue factor can also function as a transmembrane signalling receptor. Tissue factor belongs to the class II cytokine receptor superfamily (Bazan 1990) and has been implicated in the induction of signalling mechanisms involved in inflammation, angiogenesis, atherosclerosis and tumourigenesis (Bromberg *et al.* 1999). The ability of tissue factor to influence cell behaviour may arise from two distinct mechanisms. Tissue factor is capable of inducing cellular signalling directly, through its cytoplasmic tails (Abe *et al.* 1999). Alternatively, the activation of a subclass of G-protein-couples receptors called protease activated receptors (PARs) 1 and 2 which mediates singalling to FVIIa or FXa (Peppelenbosch &Versteeg 2001). The exposure of tissue factor-expressing cells (fibroblast, tumour cells or keratinocytes) to FVIIa has been shown to increase the expression of several proteins such as vascular endothelial growth factor (VEGF) (Ollivier *et al.* 1998) and connective tissue growth factor (CCN2) (Pendurthi *et al.* 2000). These proteins are the key regulator of angiogenesis and tumourigenesis. Therefore, it has been suggested that the signalling mechanisms mediated by tissue factor are responsible for the regulation of cellular function including cell proliferation and migration, and cell adhesion (Ollivier *et al.* 1998, Pendurthi *et al.* 2000).

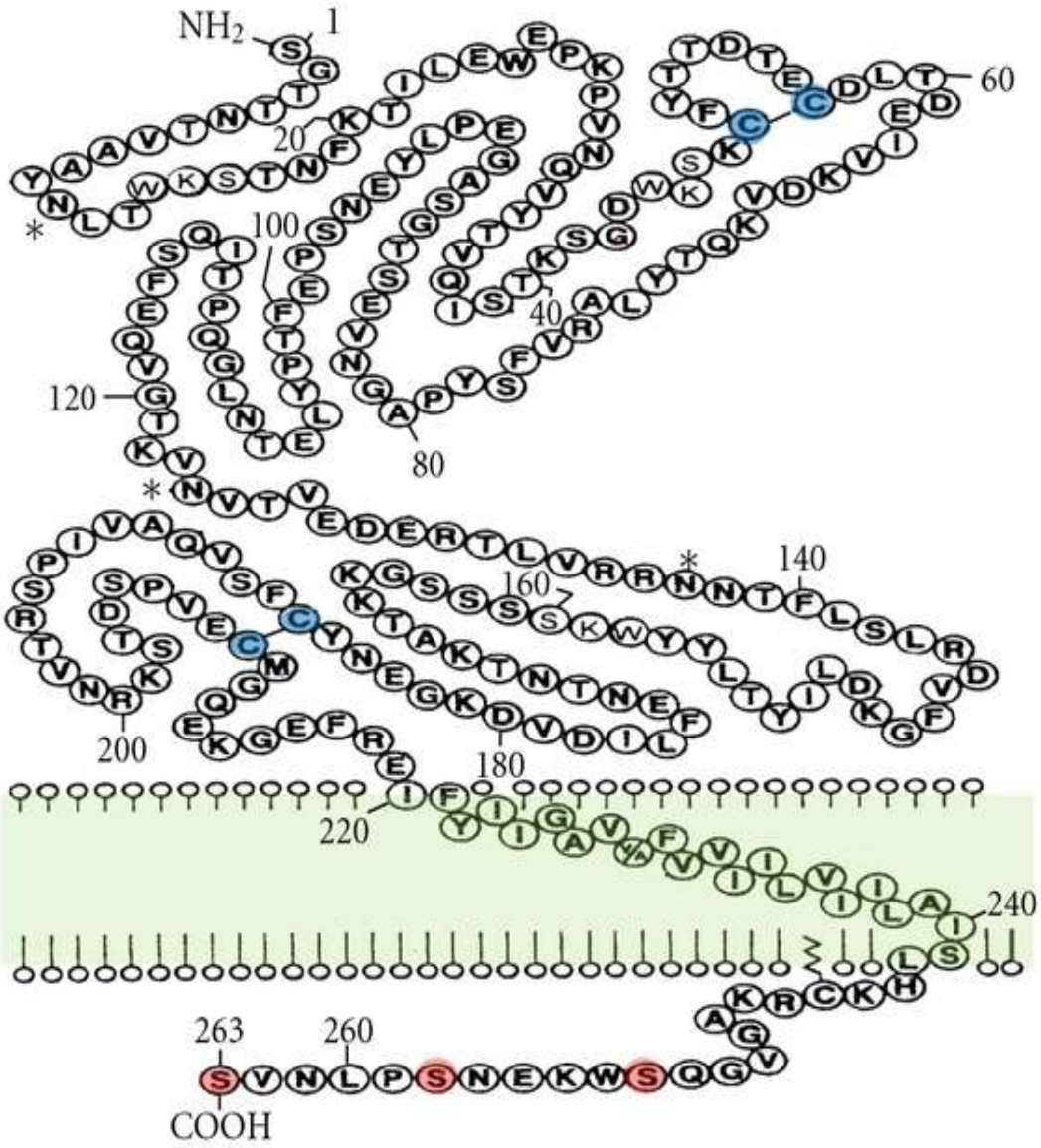

Figure 1.3: Schematic representation of the structure of tissue factor

Tissue factor consists of 263 amino acids long poly-peptide. The polypeptide is composed of a 219 amino acids N-terminal domain, a 23 amino acids transmembrane domain, and a 21 amino acids C-terminal cytoplasmic tail. [Adapted from Peterson LC *et al.* 1995.]

1.6 Transcription Regulation of the Tissue Factor Gene

The induction of tissue factor is predominantly regulated at the transcriptional level in response to inflammatory cytokines such as lipopolysaccharide (LPS), tumour necrosis factor-α (TNF- α) and interleukin-1β (IL-1β) (Scarpati & Sadler 1987) (Fei *et al.* 1993). Upon binding of agonist peptide to its receptor, the signalling cascade is initiated and phosphorylate the transcription factors such as the AP1 family transcription factors. Alternatively, activation of the signalling cascade allows the translocation of transcription factors such as the NFκB family of transcription factor, from the cytoplasm to nucleus. Consequently, transcription factors bind on to the tissue factor promoter. This in turn results in the transcription of tissue factor gene. The tissue factor promoter has been shown to consist two AP1 sites at -217 bp and -204 bp (5'-TGAATCA-3'), a NFκB sites at -179 bp (5'-CGGAGTTTCC-3'), four SP1 sites at -57 bp, -70 bp, -96 sites, and -131 bp, and a EGR1 sites at -71 bp (5'- CCGGGGGCGG-3') for the binding of transcription factor (Parry & Mackman 1995, Mackman 1995) (Figure 1.4). The AP1 transcription factor family includes Fos and Jun proteins while NFκB/ Rel family transcription factor includes p50 (NFκB1), p65 (Rel-A) and Rel-C. These proteins form homodimers or heterodimers that specifically bind to the AP1 and NFκB recognition site, respectively, which in turn induces tissue factor transcription (Mackman 1997). For example, endothelial tissue factor is induced when exposed to TNF-α. Upon binding of TNF-α to the TNF receptor, the MAP kinases p38 and C-Jun terminal NH2-kinase (JNK) signal transduction pathways are activated. Consequently, these signal events stimulate the phosphorylation of Fos/Jun proteins. Ultimately, the Fos/Jun proteins bind to AP1 transcription site on tissue factor promoter, resulting in the upregulation of tissue factor mRNA in the endothelial cells (Mechtcheriakova *et al.* 2001, Mackman 1997).

In addition to transcription regulation, tissue factor expression is modulated on the post-transcriptional level by alternative splicing (Eisenreich & Rauch 2010). Alternative splicing of the tissue factor gene results in exon 5 exclusion, causing a shift in the open reading frame. Translation of this mature mRNA splice variant leads to the expression of soluble alternative spliced tissue factor (Srinivasan & Bogdonav 2012). The expression of alternative spliced tissue factor was shown to be regulated by serine-arginine (SR) proteins splicing factor family such as SRp 40, and SC 35. The SR protein act as a competitive agonist and bind to the RNA recognition motifs site to promote exon 5 skipping, thereby facilitating the expression of alternative spliced tissue factor (Chandradas *et al.* 2010). In contrast to the high pro-coagulant property of tissue factor, alternative spliced tissue factor exhibit a lower pro-coagulant activities. Instead, the alternative spliced tissue factor is linked more closely to cell survival and pro-angiogenic process.

More recently, it has been reported that microRNA was also involved in regulation of tissue factor expression on the post-transcriptional level (Eisenreich & Rauch 2013, Zhang *et al.* 2011, Yu *et.al* 2013). microRNA is a small non-protein-coding RNA consists of 20-25 nucleotides. microRNA regulate post-transcriptional gene expression by binding to the regulatory 3'-untranslated region (3'UTR) of a target mRNA. This consequently leads to the inhibition of mRNA translation or promotes mRNA degradation (Fukushima *et al.* 2011, Kawashima *et al.* 2011). Moreover, microRNA-mediated expression regulation was suggested to play an important role in various physiologic and pathologic process such as inflammation, tumour growth and thrombosis (Eisenreich *et al.* 2010). A study by Zhang *et al.* (2011) showed that over-expressing of microRNA-19 in MDA-MD-231 cells significantly reduced the expression of tissue factor. In contrast, a high level of endogenous tissue factor was

detected in MCF-7 cells when microRNA-19 was suppressed with a inhibitor. Furthermore, deletion of microRNA-19 binding sites on the 3'UTR of tissue factor gene results in the up-regulation of tissue factor in MCF-7 cells. It is noteworthy that tissue factor is known to be highly expressed in the invasive breast cancer cell line, MDA-MB-231 but barely detectable in the less invasive breast cancer cell line, MCF-7. Therefore, Zhang *et al.* (2011) study suggested that microRNA-19 targets the 3'UTR of tissue factor gene, consequently down-regulate the tissue factor expression. In addition, Eisenreich *et al.* (2013) demonstrated that selective inhibition of microRNA-19 and microRNA-126 in human microvascular endothelial cells (HMEC-1) reduced the binding of these microRNA to the 3'UTR of the tissue factor gene. Subsequently, tissue factor expression within HMEC-1 was induced. Taken together with the above studies, it has been suggested that microRNA are involved in the regulation expression of tissue factor in different cells such as endothelial cells and cancer cells (Eisenreich *et al.* 2013, Zhang *et al.* 2011). Since tissue factor has been reported to be pro-angiogenic, microRNA-mediated tissue factor expression may directly affects the tissue factor-associated cellular functions such as cell thrombogenicity and cell migration (Yu *et al.* 2013).

```
3' -798       GAATTCTCCCAGAGGCAAACTGCCAGATGTGAGGCTGCTCTTCCTCAGTCACTATCTCTGGTCGTACCGGGCGATGCCTGAGCCAACTGACCCTCAGA
  -700  CCTGTGAGCCGAGCCGGTCACACCGTGGCTGACACCGGCATTCCCACCGCCTTTCTCCTGTGCGACCCGCTAAGGGCCCCGCGAGGTGGGCAGGCCAAGT
  -600  ATTCTTGACCTTCGTGGGGTAGAAGAAGCCACCGTGGCTGGGAGAGGGCCCTGCTCACAGCCACACGTTTACTTCGCTGCAGGTCCCGAGCTTCTGCCCC
  -500  AGGTGGGCAAAGCATCCGGGAAATGCCCTCCGCTGCCCGAGGGGAGCCCAGAGCCCGTGCTTTCTATTAAATGTTGTAAATGCCGCCTCTCCCACTTTAT
  -400   CACCAAATGGAAGGGAAGAATTCTTCCAAGGCGCCCTCCCTTTCCTGCCATAGACCTGCAACCCACCTAAGCTGCACGTCGGAGTCGCGGGCCTGGGTGA
                                                                                                  AP1       AP1
  -300  ATCCGGGGGCCTTGGGGGACCCGGGCAACTAGACCCGCCTGCGTCCTCCAGGGCAGCTCCGCGCTCGGTGGCGCGGTTGAATCACTGGGGTGAATCATCC
                      NFκB                                                    SP1                       SP1
  -200   CTTGCAGGGTCCCGGAGTTTCCTACCGGGAGGAGGCGGGGCAGGGGTGTGGACTCGCCGGGGGCGGCCCACCGCGACGGCAAGTGACCCGGGGCGGGGGC
                                SP1       EGR1      SP1
  -100  GGGGAGTCGGGAGGAGCGGCGGGGGCGGGCGCCGGGGGCGGGGAGAGGGGCGGGAGAGCGCGCCGCCGGCCCTTTATACCGGGGCGGGGACCGGCTCCCC

                                                  Exon 1
     1  AAGACTGCGAGCTCCCCGCACCCCCTCGCACTCCCTCTGGCCGGCCCAGGGCGCCTTCAGCCCAACCTCCCCAGCCCCACGGGCGCCACGGAACCGCTC
   101  GATCTCGCCGCCAACTGGTAGACATGGAGACCCCTGCCTGGCCCGGGTCCCGCGCCCCGAGACCGCCGTCGCTCGGACAGTCCTGCTCGGCTGGGTCTT
   201  CGCCCAGGTGGCCGGCGCTTCAGGTGAGTGGCACCAGCCCCTGGAAGCCCGGGGCGCGCCACACGCAGGAGGGAGGCGACAGTCCTGGCTGGCAGCGGGC
   301  TCGCCCTGGTTCCCCGGGGCGCCCATGTTGTCCCCCGCGCCTACGGGACTCGGCTGCGCTCACCCAGCCCGGCTTGAATGAACCGAGTCCGTCGGGCGCC
   401  GGCGGGAGTTGCAGGGAGGGAGTTGGCGCCCCAGACCCCGCTGCCCCTTCCGCTGGAGAGTTTTGCTCGGGGTGTCCGAGTAATTGGACTGTTGTTGCAT
   501  AAGCGGACTTTTAGCTCCCGCTTTAACTCTGGGGAAAGGGCTTCCCAGTGAGTTGCGACCTTCAATATGATAGGACTTGrGCCTGCGTCTGCACGTGTTG
   601  GCGTGCAGAGGTTTGGATATTATCTTTCATTATATGTGCATCTTCCCTTAATAAAGAGCGTCCCTGGTCTTTTCCTGGCCATCTTTGTTCTAGGTTTGGG
   701  TAGAGGCAATCCAAAAGGGCTGGATTGCTGCTTAGATTGGAGCAGGTACAACGTTGTGCATGCCCCGTATTTCTACGAGGTGTTCGGGACGGCGTAGAGA
   801  CTGGGACCTGCTGCGTACTGGCAAAGCAGACCTTCATAAGAAATAATCCTGATCCAATACAGCCGACGGTGTGACAGGCCACACGTCCCCGTGGGTCTCT
   901  GTGGAAGTTTCAGTGTAGCGACATTTCAGATAAAAGTGGAAAAAGTGAAGTTTGGCTTTTTTCATTTGTATGCAGTCCTAACTCTTGTCACACGTGTGGG
  1001  ATTTATCTTTTTCCATAACTTACTGAAAACCCTTCCTGGCGGGCTGAACCTGACTCTTCCTGAGCTGAGTCCTGGACTGGCACACTGATGGCTCTGGGCT
  1101  CTTCCCGGTCAAGTTATAACAAGGCTTTGCCCATGAATAATTTCAAACGAAAATGTCAAGATCCTTGCCGGTGTCCTGGGATTACAAGGTGAATCTTGTC
  1201  ATGAAGAAATTCTAGGTCTAGAAAAAATTTGAAGATTCTTTTTCTCTTGATAATTCACTAATGAAGCTTTTGTGGTTGAAAAATAAAAAGTGAGGTTTAT
                                                                                                  Exon 2
  1301  GGTGATGTCAGGTGGGAAGGTGTTTTATACATCAATACATTCGAGTGCTCTGAAGTGCATGTAATAATAGCTGTTTCTCTGTTGTTTAAAGGGCACTAAA
  1401  ATACTGTGGCAGCATATAATTTAACTTGGAAATCAACTAATTTCAAGACAATTTTGGAGTGGGAACCCAAACCCGTCAATCAAGTCTACACTGTTCAAAT
  1501  AAGGTAAGCTGGGTACAGA AAAAGAAAATTAAGGTCTTTGATGTTTCTACTGTCCTATGCTGAACAAGAATGTCTTTAAAGCTGATTACTGGATGAA ATT
  1601  ATTTAACAGATGACGAAGAAGAAGGGATTCTTGGCAATTCGCTGGCCGGTGTCATACTCTATTAGGCCTGCAACATTTCCAGACCTTAAACTGATAGAAC
  1701  ATTTTAATTGTTTTAATTGTTTTTGGAAATGATGGGAGAGTTCCTAAGTGGAGTATAAACTGTGGAGAGATGAACCATCTTGAGTAGGCACTGAAGTGTG
  1801  CTTTGGGTCATGATAGATTAATTAATCTCATCTAAACATTGATGTCTTTTTCCGTTGCTGTCTAGACTGTGAACAATGTCTAACACCTTAGGGAAGAGGT
  1901  GGGGAGGAATCCCAATGTATACATTGCCCTTAAGCAGTGTTTGATTCATTCATCTTTGGACTCCATGAATCGAAATCTGGTAGAATACATGATCTTAGTG
  2001  GAGGAGGCCAAATGCGTGACTCACTGAGCCTGGCAGAGCAGAAATACTCTGCTGTCTGCACCCTCTGGGTCTGGTGTGGCTCTGCTTCTTGGTGCTTCAA
  2101  CTCTGACTGGCAGCTGTCCCCAGGAGGCGATAATTCAGCATGTTCAATCTAAAGGTTATGACTTCCTTGATGGTTTTCACCATATTCTTGGCAAGTTTTT
  2201  GGTTTTTGAAATGTTCTAGGAGGCTTGGTAGAGATCTTATGAAATAGAGAATAGCTGCTGTGGAAATTATTTTAATGCTAATTACATAAAAGTACAAAAG
  2301  TAGCACTAGCTAAAACAAAAGGTATTTTGCTGTTCTGTTTTGTTTTAGCTTGTGCCAGGCCTTTTACAGCATTAGGAATGCAACTTCTAGATAACGATGC
  2401  ATCTTTTAAGTGAATGTTCTTGTTTTTCAAAATGAACTTCATGACAGTAGTTGCCAAACCAGCAAGGAGAACTTGCATGCATACGTGCATGCATGTGTGG
  2501  ATATGTATGGGGGTGGGGGGAGAGAAAGATGAAGGAATTTCATAACATGAAATAATGATTACAGTTCTGGTCAAACTTGTCAATTCAGATTTCACCAATT
  2601  GAGAATTAGTAAGTAATTTCTCTGATACAGGCCTGAAGTTTACCTTAGTAAACACTTTACTTCCATATGGTAAAAATTAGATTTTGGGAGGAATGCTTAC
  2701  CTCCTAAATATATTCAATCTAATATTTGAGGACACATGGGAATATATTTATGATTCATCTGCTTTTTAAACATAAGCCTTTGTTAACTGTAAGTTCTTGA
  2801  ACTTTATAAGGCTGCTGTTATTTAAATGAGCACAGCTCCTGATCTGCAAACAGCAGAGCGCAGGGCTACAGCTTGGGGGATGCCAGCCGACTCAGGGTGG
  2901  TCCTGTGGACTGAACAATCTCTTGCTGCTGTACTGGAGGGCCTGGGAGCTTTTCCATCAGCTCGGCCTGAGGTGTGCACTCTTCTCCTGCCCACCCCAGA
  3001  GAATAAATGAGATTCCTGGTTAAAAAGGACCAGAGCAGTCATTTTACAGTTGAGGAAACTGTTGCTCTGAGAAGTGAGGGATTTATTCATGACTACACTG
  3101  ATGGTGAGTGCCCATGTCAGGTCTGGAACCAAAGTCTACCCAGTATCCACACACCACCATCCCTCAGGTGGCTCTGCCACAGTCTGATGGGAGGCTCCAA
  3201  TCTCTTCCCTCCCGACTGCTCTCACTACTGCAGCCTGGCTCCAGCCGCAGGACACTACTGCTGTGCAGAAGCCCCTACTTGGAACTCCAACTGCATTTTT
  3301  TCTCTTCCCTCCCGACTGCTCTCACTACTGCAGCCTGGCTCCAGCCGCAGGACACTACTGCTGTGCAGAAGCCCCTACTTGGAACTCCAACTGCATTTTT
  3401  CACCTTTGCTAACAGTTTTCAGTGGTGGTTGGGAAATGTTATTGGCTTAAGCCTTAGCACAAACCGTCACCGGTGATATrCATTCCATGGAAATGTTCTG
  3501  AATTCTAAAGCTGAATTTACAAAGCTTCTGGAAAACAACCTGCAACCAAATTAGTGACTGAATTTTTTAGTTAACTCAAAATTCCAAATCAGAGGGTTTT
  3601  GCAATGCCTGGAGGAACCTTGGAGGCTTTTAAAGTGTTAATGCTATTAATGGCATTCAGAGGGATTTTCTACAGAATTGTCCCTTCATTACCTGTTTATA
  3701  CAGTTTTACTACTTACCAGGGTACTGTATAAATCCTTGTGCTAAATTTTGCTATAGAGTATGTGGTCCCTGCTGTGAGCTGGGAGGAACCAAATACTGTA
  3801  TCTCTATGTTACATAGAAAGCCCTAGGAGACTTTCTCCTGTTATCTGAACAACTATTTGCTGTACTGATAAAAAGGAAACAGCATAGTCTCATTCACTTT
  3901  TTGAAATGGAAATGATAAAATAAAACACATTTTGGTCATTCGGGAACAAAATACCCTCTCTACTTTTATCACATAAAATrAAATAAATAGAAACCAAAAT
  4001  ATTTCAGTATCAATCTTAGTTTGTGCACTTTAGGATAAAGAATGTGTTTACCCAAATCCTTTTGGCCTGGTTACTTAGTrCAGATTTTGAAAGAAAATAT
  4101  ATTTGTGGCTTTTATGTGTGAATTTAGACAATGGAATCCATGTGGTGCCTCGTTTTCCCTGAGATTATGTATTAATTCAACCTGTAAATGCAAACCATCT
  4201  AATAGTCAGCGAGACCCTATAGCCCTGCTGCTTAATGGGGGcACACAAGGGCATGCAGCCCTCGTACCAGGCAGACTGTGTTCATATTAACAGCATCGTG
  4301  GAGAAACTCATGCTGGGGGACAGGGGAGGGAGATGTAAATGCTCAGCAGGGAGATCTGGAGATTCCTGGAGCAGGTGGAGTTGGGACCTGGCCTTGAACG
  4401  ATGGGTCTGGCTCTGGCAGTCAGTAATGCCAAAGGGAAGAGCAGCATAACTGTCACTTTCCATGGGACAGAAGTGTGTGAATCAAGTTGCAGTGACGCTT
  4501  CACCTATTTATTATTTTGGTCATTTAGAAGAATTTCATTGTCAGTAGAAGTCCTTTAAATCATTTCCCCTTCAGTGACGTCTCACAAAAAAAAGATCTGT
  4601  CTTTAGCTTTTTAGTCTCAGACTTTATTAGACAGATACTACCTGTACTCTTATTCTGTAATCTTTGTTGGGATGGATTCACATCTTGCAAAGGAAGGGAG
  4701  GCATGTAGTATAATGGGGCAAACAGACCCAGCTCTGCCACTCGTTAGATATGTGACCTTCTGCAAGTTGCTTAGTGCCTGTGAGCTTCAGTGTCCTCATG
  4801  GATAAGAAAGATCCAACACCTTCTTGGAAGGATTATATCAAATGAAGTAACATGAGTAAAGGGTCCAGCAGAATACCTGGCATATAGTGGAGTCAATGAA
  4901  TGATTAATAATATTATTAATAGTGGTCATGAGAGATATATGTATAACATGTTATTATGTAGACTCACTATATAGACTCTATTCTACATAGAATATAGAAC
  5001  ATTATATAACAAACAACTATAATAAGTAGACTATAGTAAACAACCTCACTTTGTCTCAGTTGCCTCATCTTGATGGAAAACTGCTCTTTCTCTCCTGTTA
  5101  CCCTGACAGAGAGCGTCTACATTCTAAAAGAAAGATATTTAACAAAATGGTTGAGTACAGATCCAAGAGTCAAATAGCTGTCTGGTTCAAAGTCCAGCTG
  5201  TGTGATTTTGAGCTAGTCACCCAATCTCACTTTGTCTCAGTAGCCTTATTTGTAAAAACAAGGCAAATTACAGAGCCATCCCCTGGGTTGCTATGAGGAC
  5301  TCAAACATGCATCCCAAGTGCTCGGTGTTGCTAGGTATGATGGCTCACACCTGTACATTCAGCACTTTGGGAGGCCGAAGcAGAAGGATCAGCCTGGGCA
  5401  ACATAGCAGGACCCCATCTCTACAAAACAATGTTTAAAAAAAAGCAAAGTGCTCAGCACAGTGACTGCATCATTAGGATrGATTGTAGGGCTCCTGATGT
                                                                                                  Exon 3
  5501  TAGCACAGAACACCACAGCCAGGAAGCAGTCTATCTTGTTGGGTGCAAATTGTAACATTCCATTTATGTTTCTTCCTTCTTTTCTTTCTTTAGCACTAAG
  5601  TCAGGAGATTGGAAAAGCAAATGCTTTTACACAACAGACACAGAGTGTGACCTCACCGACGAGATTGTGAAGGATGTGAAGCAGACGTACTTGGCACGGG
  5701  TCTTCTCCTACCCGGCAGGAATGGTGGAGAGCACCGGTTCTGCTGGGGAGCCTCTGTATGAGAACTCCCCAGAGTTCACACCTTACCTGGAGAGTAAGTG
  5801  GCTTGGGCTGTAATACCGTTCATTCTTGTTAGAAACGTCTGAACATTCTCGTGATCTTGTGCCTTTAGGGGCTACAAAATTAAAAATATTTATTCTTTTT
  5901  TTCTCAGAAACTGGTATGTATCACAGCCCTCTTCACACATTCCAGATGTGGTAGGAGGTTCACAGAATGTGAACTTTTGGAGCTGATGACAGTGTCATCA
  6001  AGTAACTTTCTCCCCCAGTCTGTCCCCAGACCCTGTTACTGTCCTCAGTAAGCGGCTGAATGTGTGTTGGGAGAGGGCGGGCCAGGGAAGCGGGTAGGGA
  6101  TAGGAAATCCACCAAGGCCGGGGTTTTAGCTTTTCCCTATATATATATCATGTATCCTGATTTTTCTGTCCCGTTATCACACTAAAAATCCCAGTTGAGG
  6201  ATTTTTCCCAAACGGTCATAAATCAATGAGGAAAGTCCATGGTTTCCCTCTGAGCCCATAATTAGCCTAATTATGCTGACCTTTTCTAATCAGTTGGCCA
  6301  TGATTTGAGTTCCGTGATGTGCCAGCACCTGCCCAGCCATCTGCCTGTCACCCTCGTTCTGGTTTTGGAAAGGTGGAATACTTTCCTCCTCAGCCTTTGC
  6401  CCCTGTAAGCTGGCCCTAGGAGCCAGTAAAAGAATGAAGAGAATTCCTGTCAAGTAGGAGATTTATTCTTTTGCCGCAACTGTGGCTCTGAGCTAGGCAA
  6501  TTTAGATAAATGCATGTAGCACATTGAGTAGAGTGAAATTAGCTTCTCTTGTAAGGCCAGCTGGTTAGAATGAAGGTGTTGTGTGAGTGTTAGGCCCAGC
  6601  GAGAGAGAACAGTTTCTCAAGGTAGGAATGGTGAAAAGAAGGGGTGGACGGACAACCAACCAACCATCCTCCTCTGGTArCTACTTTGAGGGTTGAAATA
  6701  GGGGGCCTGACCCCAGGTGAATGTGGCTGCCTTCCCAGACCCCCCATTTGCAAGACCCTCCAGACCCCCAGGTGCTTCT9CTTGTGTCTTTTGTGGCACC
  6801  AGGCAAGAATGTAGCAGCGTCAGCAGCCCCTCTGGTGACTGTGGCATGGTTGACATTCATTTCCCCCCTAATTAATGGCATCCTCATGATTCTCTTTTAT
  6901  ATTAATAGTTCTTGAGTTTTTTTGTAAGCTACTTCAAATCCTTTGTTGGTGCAAGATAGAAGATATTTTATGTGTTTGTTTTGCATGTGCACACACATAT
  7001  TTGGCCTGTGAATTGATGTTTGTTTTCCTGTCATTAACCAAAGCACATGAGATAATTGAGCCATTGCAGAGACCCCGTGGTTAAATCCGGCTTCTCGAG
  7101  GTACCAAGGACATTTCCTGGGTTTTCTCACAGCCCTACATATTTTTGAACCTAAAATATCGTAGTTTATGCTACCACCCTGTTCAGTATAGTAGCCACTA
  7201  GCCACATGTGGCTGTTGACCACTTGAAATATGGCTAATGCTCTAAGTATAAAGTACACACTGGAATTTAAGAAGTGTAGAATATCTCAAAACTTTTTTAT
  7301  ATTGATTACACATTAAAATGATTATATTCCAGATATATGCAGTTGACTCAAGCAATGCATGGCTGAGAGGCACCGACTCSCTGTGCAGTTGAAAATCCGA
  7401  GTATAACTTGACTCCCCAAAAACTTAACTACTAATAGCCTACCTATCGGTTGACTGTTGACTGCAGCCTTACCAATAAGATAAACAGTCAATTAACACAC
  7501  ATTTTTCATGTTGCGTGTATTATATACTGTATTCTTACAATAAAGTAAGCTAGAGGAAAGAAAATGTTATTAAGAAAATTATAAGGAAAAGAGGCTGGGC
  7601  ATGGTGGCTCGTGCCTGTAATCTCAGAACTTTGGGATGCTAAGGCGGGTGGATCACTTGAGGTCAGGAGTTCAAGACCAGCCTGGCCAACATGGTGAAAC
  7701  CCCATCTCTACTAAAAATACAAAAATTAGCCAGGCGTGGTTGTGGGTGCCTGTAATCCCAGCTACTTGGGAGGCTGAGGCAGGAGAATCACTTGCGACCCA
  7801  GGTGGAGGAGGTTGCAGTGAACTGAGATTGCGCCACTGCACTCCGGCCTGGGTGACAGAGCGAGACTCTGTCTAAAAAAGAAAGGGAAAGAAAGAAAAAA
  7901  AAGAAAAGAAAAGAAAAGAAAGAAGGAAGGAAGAGAAAGAATTATAAGGAAGAGAAAATATATTTACTATTGATAAAGTGGAAGTGGATCATCATAAAGG
  8001  TGTTCATCCTCGTCATCTTCATGTTGAGTAGGCTGAGGAGGAGGAGGAGGAAGAGCAGGGGCCACGGCAGGAGAAAAGATGGAGGAAGTAGGAGGCG
```

8101 GCACACTTGGTGTAACTTTTATTTAAAAAAATTTGCATACAAGTGGATCCACAGAGTTcAAACCCATGTTGTTCAGGGGTCAACTGTCTTTGGTTAAATA
8201 AAATATATTATTAAAATTAATTTCACCTGTTCCTTTTTACTTTTTCTAATGTGACTACTAGAAAACTTAAAATGACATCTGAGGCTCCATTGTCTTCCCC
8301 TTGGGCCAGCACTACCACAGAATGTCTTAGGATTCAGCTCCAGGCCGCCACGCCTGCTTCTTTCAGGGAGCTGGTTCTATGCACATGTTTTATATGAGAG

Exon 4

8401 ATAATTAAGTTGTCAATTGTGATAACAAAACAGGATTTGACTTTGTACAGAATTCTTTGGTTCCAACCAAGCTCATTTCCTTTGTTTCAGCAAACCTCG
8501 ACAGCCAACAATTCAGAGTTTTGAACAGGTGGGAACAAAAGTGAATGTGACCGTAGAAGATGAACGGACTTTAGTCAGAAGGAACAACACTTTCCTAA
8601 CTCCGGGATGTTTTTGGCAAGGACTTAATTTATACACTTTATTATTGGAAATCTTCAAGTTCAGGAAAGGTGAGCATTTTTTAATTTGTTTTTATGACCT
8701 GTTTTAAATTGTGAATACTTGGTTTTACAACCCATTTCTTCCCCAATTCAAAAATAGCAGAACAGAGTTGTTGAGAAGGTGATGGAGTAGAAGGGGGAGC
8801 GCGCACTGTGGGGAGGGGTGGACAACAGGCCTGGTCCTAOcTGTGACTCTGCACTACCCTGTGACTCTGGCAGGGCCCCcCTCGGAGACCCAGGTTCCTCA
8901 GCCAACCGGCTGGATCAGGTCATCTCTAAAGGTCCCGCCACGCTCACATTTCTCCCTCTATTGAGGATCCCAGGCACAAAATTTGTTTTTGGTTCAATGC
9001 ATAATACTCCCTTCCTTTrTCTTTTACTGCAGATATCTTCTAAAGGGGCTCAATAGGGTTCAATATGCCTAAATTGGATCTTCTCAGTCTTGGAAAAGGC
9101 ATTTTTAGCAGTGATCAAGGGAAACTGATTAGCGAAGTCACTTCTAATCCTTCACGTGTCAGCTGTGTTCTTGTAGGCTTTGCTTAGAACCTAGGTTTTT

Exon 5

9201 ACTTCCACAGTGACTTAATAAAGGGGAAAGAATTGACTCAGAGCCCAGATGAATTAAGAACTCTATCTTTTTACAGAAAACAGCCAAAACAAACACTAAT
9301 GAGTTTTTGATTGATGTGGATAAAGGAGAAAACTACTGTTTCAGTGTTCAAGCAGTGATTCCCTCCCGAACAGTTAACCGGAAGAGTACAGACAGCCCGG
9401 TAGAGTGTATGGGCCAGGAGAAAGGGGAATTCAGAGGTGAGTGGCTCTGCCAGCCATTTGCCTGGGGGTATGGGTGCTCGTGGGTGACTTCTGGAGGAGTA
9501 GCTCCACCCTCAGGGCTGGGATATACTTCCTTGGTTAAATATTCAGGAAAACAAACTGCCTGGAGGTTTTTTGTTGTTATTTGTTTGTTTTGGTTTTGAT
9601 TTTGCTTTGGTACAAAAAAGATTTTGGACATTTAGAAATGrTTCTGTGTTGATTGTGCCCTTGTATTAGCAGGTGTTTTCTTGAGCACCTGTCATGTGCT
9701 AAGCCCTCTGCTGAGCACTGGATACACAAACTGTGTTTAGGATTTAGCAACAAGTCACAGATTTCCCTGGGCATTTTTrCATGCTTAAATTCTAATTCTG
9801 GGGGTGGCTTCTGGACCAGCTGCAACAGGACACAGTAGACATTCGTGAGTACCCACTGTGGGCTGTTGCCACAGAGGCTGTAGAGTCTAACCCATCAAGG
9901 GAAGGGATTGAGTATATCAAATATACCCACATGCATGCATGTGTGTATATGGCGGACACGTGTGTGTACATGCATGTGCATATGTTGGGAGCTCAGGCCC
10001 ATTGTGCGAGGAACAGTCCCTAACCGGAAGTGCTGTGGGCCTTCAGACTCTTGCAGGAAGCTGCAAGCCTGTGTGTCTCGATCCATGCCTTACAGGGAAA
10101 GTATTCTGAGTACTTTCAGTGAAGAAAAGAGTCAGGGGATATAAACGATGGCTTACGCTGGGTGTGGTGGCTCACGCCTGTAGTCCCTGCACTTTGGGAG
10201 GCCCAGACAGGCAAATCACTTGAGGTCAGGAGTTTGGGACCAGCCTGGCCAACATGGTAAAAGCCCATCTCTACTCAAAATACAAAAAGTAGCTGGGTGT
10301 GGTTGCACGTGTCTGTAGTCCCAGCTACTCAGGAGGTTGAGGCAGGAGAATTGCTTGAACCTGGGAGGCGGAGGCTGAAGTGAGCTGAGATTGGACCACT
10401 GTACTCCAGCCTGGGTGACAGAGCGAGATTCCATCTCAAAAAAAAAAAAAAAGAAACAACGAAAAAAGAAATGATGGCTTAGCTCCATGTGAAGATGATAT 10501
TTGAACATTTTAAAACACTTTAAATAAACTGTTCTCTCCTGTTTATTGCCACTGACAGGAGAGGTTTCTCTTTACCTCTGGTCCTGCACCCCTCTGAGCC
10601 ATCCTACCCACAGCCTTCAGTCATTGTCCTAAAGCCTAGCTCTAATTCCACTGCCTCTCCTTTTGTGCACACACACTTCTCTGCTTCCCTGGCCGTTCTC
10701 TATCTTGGAGAGGCATTTCAAACGCCACTTCCACCAGAAGGcCTTGCTACTGCACCAACTAGTTACTATCTCTTCTTCACcCAAATCCTGGTAGCACTTT
10801 GGATCTCCCACTTGCACTTAGGGTTCACCTTCCGTTATAATCATTGCCATCAATCTCAGCATCGTTTTAGGCACTTCTTTCCAGCCATTGTTCTTACCTC
10901 CAACTACATATCTTTTCTGGACTGTGCATTATTCAGTTTATTAAATGCCCATTAAATGTGTTTAGCCATTGTCAATTACTCTGAAACGTTCAGGTTTTGA
11001 CAAATTCTTTCCTAATGTAAGTGTGGTGGAAAGAGTGAAAGAAAGTCAAATTGCACAAAAATAGGATGGTGTAATTTGGGGTTATGCCGTCAATTTTGT

Exon 6

11101 CACTGATAAATGGGATTTGAGCTCTCCAAGTTGACTAGATGCCCTTTATTTTTCAGAAATATTCTACATCATTGGAGCTGTGGTATTTGTGGTCATCATC
11201 CTTGTCATCATCCTGGCTATATCTCTACACAAGTGTAGAAAGGCAGGAGTGGGGCAGAGCTGGAAGGAGAACTCCCCACTGAATGTTTCATAAAGGAAGC
11301 ACTGTTGGAGCTACTGCAAATGCTATATTGCACTGTGACCGAGAACTTTTAAGAGGATAGAATACATGGAAACGCAAATGAGTATTTCGGAGCATGAAGA
11401 CCCTGGAGTTCAAAAAACTCTTGATATGACCTGTTATTACCATTAGCATTCTGGTTTTGACATCAGCATTAGTCACTTTGAAATGTAACGAATGGTACTA
11501 CAACCAATTCCAAGTTTTAATTTTTAACACCATGGCACCTTTTGCACATAACATGCTTTAGATTATATATTCCGCACTCAAGGAGTAACCAGGTCGTCCA
11601 AGCAAAAACAAATGGGAAAATGTCTTAAAAAATCCTGGGTGGACTTTTGAAAAGCTTTTTTTTTTTTTTTTTTTTTGAGACGGAGTCTTGCTCTGTTGC
11701 CCAGGCTGGAGTGCAGTAGCACGATCTCGGCTCACTGCACCCTCCGTCTCTCGGGTTCAAGCAATTGTCTGCCTCAGCCTCCCGAGTAGCTGGGATTACA
11801 GGTGCGCACTACCACGCCAAGCTAATTTTTGTATTTTTTAGTAGAGATGGGGTTTCACCATCTTGGCCAGGCTGGTCTTGAATTCCTGACCTCAGGTGA
11901 CCACCCACCTTGGCCTCCCAAAGTGCTAGTATTATGGGCGTGAACCACCATGCCCAGCCGAAAAGCTTTTGAGGGGCTGACTTCAATCCATGTAGGAAAG
12001 TAAAATGGAAGGAAATTGGGTGCATTTCTAGGACTTTTCTAACATATGTCTATAATATAGTGTTTAGGTTCTTTTTTTTTTCAGGAATACATTTGGAAAT
12101 TCAAAACAATTGGCAAACTTTGTATTAATGTGTTAAGTGCAGGAGACATTGGTATTCTGGGCACCTTCCTAATATGCTTTACAATCTGCACTTTAACTGA
12201 CTTAAGTGGCATTAAACATTTGAGAGCTAACTATATTTTTATAAGACTACTATACAAACTACAGAGTTTATGATTTAAGGTACTTAAAGCTTCTATGGTT
12301 GACATTGTATATATAATTTTTTAAAAAGGTTTTCTATATGGGGATTTTCTATTTATGTAGGTAATATTGTTCTATTTGTATATATTGAGATAATTTATTT
12401 AATATACTTTAAATAAAGGTGACTGGGAATTGTTACTGTTGTACTTATTCTATCTTCCATTTATTATTTATCTACAATTTGGTGTTTGTATTAGCTCTAC
12501 TACAGTAAATGACTGTAAAATTGTCAGTGGCTTACAACAACGTATCTTTTTCGCTTATAATACATTTTGGTGACTGTAGGCTGACTGCACTTCTTCTCAA
12601 TGTTTTCTCATTCTAGGATGCAAACCAATGGAGAAGCCCCTAATTAGATCAGGGCAGAGGGAAAAACAAAAAACTGGTAGAAACCGGCAACCACAGCTTC
12701 AAGCTTTAAGCCCATCTCCTACACTTCTGCTCTGTACGTGCCCATTGTCACTTCTGTTCACATGCTACTGTCCCAAGCAAGTGACCAAGCCTGACAATAC
12801 TTTGTCTACTGGAGTCACTGCAAGGCACATGACGGGGCAGGGATGTCGTCTTACAGGGAAGAGAAAAGATAATGCTCTCTACTGCAGACTTGGAGAGATT
12901 TCTTCCCATTGGCAGTAGTTTGACTAATTGGAGATGAGAAAAAAAGAAACATTCTTGGGATGATTGTATTGAAACAAAATTAGGTAAAAGGACAATATAG
13001 GATAGGGAGAGATATAAGTGGAATGAGATCTCTAGAGTCCATTAAAAGCAAGCTAGATTGAGAGCTC - 5'

Figure 1.4: The complete nucleotide sequence of human tissue factor gene.

The complete sequence of human tissue factor is shown together with a 798 bp upstream of the cap site. The transcription factor binding sites, AP1(5'-TGAATCA-3') and NFκB (5'-CGGAGTTTCC-3'), SP1 (5'-GGGGCGG-3'), and EGR1 (5'-CCGGGGGCG-3') and the coding regions (exon-intron) are labelled respectively.

1.7 The association of tissue factor bearing-microparticles with VTE

Increased expression of tissue factor has been demonstrated in many tumour cells such as breast cancer (Contrino *et al.* 1996, Ueno *et al.* 2000), lung cancer (Sawada *et al.* 1999), and pancreatic cancer (Kakkar *et al.* 1995). The expression of tissue factor in cancer cells has been shown to reach up to 1000-fold greater than that in normal cells (Rak *et al.* 2009). In addition, increased tissue factor is observed within the adjacent tumour-stroma associated with endothelial cell, tumour-infiltrating macrophages and fibroblasts activities (Rickles 2006). This augmented expression of tissue factor has been linked to the occurrence of VTE (Falanga 2005, White *et al.* 2007, Khorona *et al.* 2007). However, the level of tissue factor expression in the circulation varies among various types of cancer and is dependent on the stage of disease. Overall, patients with invasive cancers appear to have a higher level of tissue factor expression in the circulation and are generally at a greater risk for VTE (Kakkar *et al.* 1995).

Although increased tissue factor expression has been suggested to contribute to cancer-associated thrombosis (Falanga 2005, White *et al.* 2007), there does not appear to be a direct correlation between tissue factor expression by cancer cells, and the risk of developing thrombosis (Furie & Furie 2006). An early study by Dvorak *et al.* (1983) reported that cancer cells shed procoagulant plasma membranes vesicles, which are now referred to as microparticles. Dvorak *et al.* (1983) proposed that these procoagulant microparticles may account for the activation of blood coagulation system associated with malignancy. Since then, the role of microparticles in cancer-associated thrombosis has been intensely studied and a number of studies have reported that tumour cells shed tissue factor within these microparticles (Tsselaar *et al.* 2007, Zwicker 2009). Furthermore, elevation in the amount of circulating procoagulant microparticles within the plasma of cancer patients and especially those who developed VTE has been

reported (Hron *et al.* 2007, Khorona *et al.* 2008, Zwicker *et al.* 2009, Tsselaar *et al.* 2007). However, despite the various reports showing elevated levels of microparticles in the plasma of cancer patients, only circumstantial evidences are presently available for the procoagulant role of microparticles in cancer-associated thrombosis. Tesselaar *et al.* (2007) observed high levels of circulating procoagulant microparticles in metastatic breast cancer and pancreatic cancer patients who presented with acute VTE, compared to healthy subjects or cancer patients without VTE. Tesselaar *et al.* (2007) suggest that the procoagulant activity of the microparticles is due to the presence of tissue factor within microparticles. However, due to the limitations of current available techniques, tumour-derived microparticles cannot be differentiating from other cell-derived microparticles. Therefore, the source of tissue factor-bearing microparticles in cancer patients has not been clearly identified. More recently, Davila *et al.* (2008) showed that human pancreatic cancer cells which have been injected into the orthotopic mouse model released microparticles into the circulation, and these tumour-derived microparticles activated the coagulation pathway in a tissue factor-dependent manner. This orthotopic mouse model allows the identification of tumour-derived tissue factor from other cell-derived tissue factor in the host, such as monocytes, endothelial cells and platelets. Since the neutralisation of the tissue factor antigen within the mouse circulation, using a specific anti-human tissue factor antibody abolished the tissue factor activity, Davila *et al.* (2008) suggest that the tumour-derived coagulation activity in the orthotopic mouse is associated with tumour-derived microparticles. More recently, *in vitro* and *in vivo* evidence suggests that tumour-derived tissue factor-bearing microparticles can transfer tissue factor to stromal vascular endothelial cells (Yu *et al.* 2008). As a consequence, accumulation of the tissue factor within endothelial cells may result in increased local procoagulant activity and contributes to the development of VTE (Yu *et al.* 2008, Thomas *et al.* 2009). Although microparticles have been

demonstrated to initiate coagulation through a tissue factor-dependant pathway, microparticles are only thrombotic with the presence of anionic phospholipid (Tesselaar et al. 2007). The presence of anionic phospholipid significantly increases the procoagulant activity of microparticles because it facilitates the assembly of coagulation factor included factor VII, factor IX and factor X. This is due to an electrostatic interaction between the positively charged carboxyglutamic acid (GLA) domains in the coagulation factor and anionic phospholipid on microparticles membrane (Owen III & Mackman, 2011). Taken together with the above studies, it has been suggested that microparticles initiate coagulation in a tissue factor-dependant manner, in the presence of anionic phospholipid, and therefore may play an important role in the development of VTE (Tesselaar *et al.* 2007, Davila *et al.* 2008, Yu *et al.* 2008).

1.8 Aims of the investigation

While elevated levels of tissue factor detected within the tumour-associated vascular endothelial cells are associated with VTE, the source of this tissue factors still remains unknown. It has been shown that tissue factor can be transferred between different cell types via microparticles, and the accumulation of these tissue factor-bearing microparticles can increase the thrombogenicity of endothelial cells. This study aimed to examine the transfer of cancer-derived tissue factor by microparticles to microvascular endothelial cells *in vitro*. The study also evaluated the up-regulation of tissue factor expression within endothelial cells in response to these microparticles. Human dermal blood endothelial cells (HDBEC) were used throughout this study since thromboemboli can form on these intact endothelial cells. These cells were treated with tissue factor-containing and tissue factor-deficient microparticles derived from tumour cells and the potential of these cells in recycling the tissue factor protein and activity was investigated.

Chapter 2

Materials and Methods

2.1 Materials

Active Motif /My Bio Ltd, Kilkenny, Ireland

10X Phosphate buffered saline (PBS) for ELISA (pH 7.4)

Affinity Biologicals /Quadratech Diagnostic Ltd, Surrey, UK

0.2 mg Sheep anti-human tissue factor peroxidase conjugate lgG

10 mg Sheep anti-human tissue factor

American Diagnostic Inc, Stamford, USA

Recombinant tissue factor (non-lipidated)

Axis- Shield UK, Dundee, UK

Inhibitory tissue factor monoclonal antibody

Applied Biosystem /Life Technologies Ltd, Paisley, UK

Power SYBR ® Green RNA-to-C_T ^{TM} 1-step Kit (RT-PCR)

ATCC-LGC standards, Middlesex, UK

Breast cancer cell line, MCF-7

Breast cancer cell line, MDA-MB-213

BD Biosciences/Scientific Supplies Laboratory Ltd, Nottingham, UK

4 chamber culture slides

BDH Laboratories Supplies, Poole, UK

Citric acid ($C_6H_8O_7$)

Ethylenediaminetetraacetic acid (EDTA)

Beckman Coulter Ltd, High Wycombe, UK

TL-100 ultracentrifuge

Bioline Ltd, London, UK

Molecular grade agarose

Biorad Laboratories Inc, Hercules, CA

iCycler thermal cycler

BMG LabTECH, Offenburg, Germany

POLARstar OPTIMA (plate reader)

Carl Zeiss Ltd, Welwyn Garden City, UK

Zeiss LSM 710 confocal microscope

Clonetech Laboratories Inc, Saint-Germain-en-Laye, France

pEGFP-C3 plasmid

Dade Behring, Milton Keynes, UK

Innovin recombinant human tissue factor

eBioscience, Hatfield, UK

TrypLE- enzyme cell detachment medium

Fetal calf serum (FCS)

Enzyme Researcg Laboratories, Swansea, UK

FITC-conjugated anti-human tissue factor antibody

Eppendorf AG, Cambridge, UK

1.5 ml Eppendorf tubes

Microcentrifuge tubes

Fermentas Technology/Thermo Fisher Scientific, York, UK

Spectra® Multi-colour broad range protein ladder

Fisher Scientific /Thermo Fisher Scientific, Loughborough, UK

Glycine

Hydrochloric acid (HCl)

Sodium chloride (NaCl)

Sodium dodecylsulphate (SDS)

Tris-Base

Flowgen Biosciences Ltd, Nottingham, UK

Flowgen horizontal gel apparatus

N,N,N',N'-tetramethylethylenediamine (TEMED)

ProtoFLOWGel

ProtoFLOWGel resolving buffer (1.5 M Tris-HCl, 0.4 % SDS, pH 8.8)

ProtoFLOWGel stacking buffer

FMC Corporation, Philadelphia, USA

SYBR® Green I nucleic acid gel stain

Greiner Bio- One, Stonehouse, UK

96 wells microplate for ELISA

CELLSTAR® 25 cm^2, 75 cm^2 cell culture flask

CELLSTAR® 6,12,24-wells cell culture plate

Hyphen Biomed /Quadratech Diagnostic Lts, Surrey, UK

Thrombin substrate

Zymuphen-microparticles activity kit

ibidi /Thistle Scientific LTD, Glasgow, UK

35 mm µ-dish, glass bottom

illustra™, Burkinghamshire, UK

GFX™ PCR DNA and Gel Band purification kit

Invitrogen/Life Technologies Ltd, Paisley, UK

Kanamycin sulphate

Platinum Taq PCR Kit

T4 DNA ligation kit

LONZA, Verviers, Belgium

10X Phosphate buffered saline

Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L Glucose, with L-glutamine

Eagle's minimum essential medium (EMEM) without L-glutamine

MWG-Biotech AG, Ebersberg, Germany

Tissue factor primers

β-actin primers

Nikon, Kingston-upon-Thames, UK

Light microscope (Model TS100)

Fluorescence microscope (Model TS120)

Norgen Biotek Corporation/Geneflow Ltd, Staffordshire, UK

100 bp PCR Ranger

Origene – Rockville, USA

pCMV-XL5-TF plasmid

Promega, Southampton, UK

100X BSA for restriction activity

10X Tris-borate-EDTA (TBE) buffer (89 mM Tris-Borate, 89 mM boric acid, pH 8.3, 25 mM EDTA)

6X Blue loading dye

BamHI restriction enzyme

Buffer E for restriction activity

HindIII restriction enzyme

Midipreps DNA Purification Systems

TMB Stabalized substrate for horseradish peroxidase (HRP)

Western Blue Stabalized substrate for alkaline phosphate (AP)

PromoCell, Heidelberg, Germany

0.025 % (w/v) Trypsin/0.01 % (w/v) ETDA

Human dermal blood endothelial cell (HDBEC)

Human endothelial media MV

Supplement mix, endothelial growth medium MV

R&D Systems, Abingdon, UK

Escherichia coli TB-1 strain

Santa Cruz Biotechnology Inc, Heidelberg, Germany

Alexa-Fluor 647-labelled annexin V

Donkey anti- goat lgG conjugated HRP

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) goat polyclonal HRP

SIGMA ALDRICH, Dorset, UK

200 mM L- Alanyl- L-glutamine

27 % Formaldehyde

2-Propanol

Laemmli buffer 2X concentrate sample buffer

Ammonium persulphate

Bovine serum albumin (BSA)

Chloroform

Dimethyl sulphoxide (DMSO)

Donkey serum

Dynasore hydrate

Luria Broth agar

Luria Broth powder (1 % w/v Tryptone, 1 % w/v NaCl, 0.5 % w/v yeast extract)

Barium sulphate-adsorbed plasma (BSAP)

Super optimal broth with catabolite repression (SOC) medium (Tryptone 2 % (w/v), Yeast extract 0.5 % (w/v), 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20 mM Glucose)

Tri reagent

Triton X-100

Polyoxyethylenesorbitan monolaurate (TWEEN 20)

1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI)

0.1 % Cyclohexamine

3,3',5'5 –tetramethylbenzidine (TMB) liquid substrate system

Syngene Dene Tools, Cambridge, UK

GeneSnap image capture system and GeneTool software programme

Ultra-Violet Products Ltd, Cambridge, UK

3 Ultra-violet transilluminator

Webwe Scientific International Ltd, Leicestershire, UK

Haemocytometer

Whatman ® plc, Kent, UK

0.2 µm Protran Nitrocellulose membrane

WPA, Cambridge, UK

Lightwave UV/VIS diode array spectrophotometer

Yorkshire Biosciences Ltd, York, UK

Q-step 4 DNA ladder

2.2 Cell culture procedures

The breast cancer cell line MDA-MB-231 which expressed high level of tissue factor was cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and fetal calf serum (FCS) (10 % v/v) and MCF-7 which has low tissue factor expression was cultured in Eagle's minimal essential medium (EMEM) with Glutamax (1 % v/v) and FCS (10 % v/v). Human Dermal Blood Endothelial Cells (HDBEC) was cultured in Human Endothelial Medium MV supplemented with Endothelial Cell Growth supplement (5 % v/v). All cell culture was incubated at 37 °C in a humidified incubator, supplied with CO_2 (5 % v/v). The growth medium was replaced after 24 h to remove residual of DMSO and unattached cells, and every 2-3 days thereafter.

The breast cancer cell line and endothelial cells were propagated as adherent monolayer and subcultured when the cells reached approximately 80 % confluence. The required volume of fresh complete medium (1 % v/v Glutamax, 10 % v/v FCS) for breast cancer cell lines and endothelial cells (5 % v/v endothelial cell growth supplement) was prepared in advance and pre-warmed at room temperature for at least 30 mins. The old medium was removed and the cell layer was rinsed gently with phosphate buffered saline (PBS) (pH 7.2) (2 ml) to remove any remaining medium. To detach the cells, breast cancer cells were incubated with trypsin (0.025 % w/v trypsin, 0.01 % w/v ethylenediaminetetraacetic acid (EDTA) (2 ml) while endothelial cells were incubated with TrypLE (2 ml) at 37 °C for 3 min. Cell dissociation was monitored by examining the cells with a microscope. When about 90 % of the cells have rounded up, the flask was tapped gently to detach the cells and old medium (2 ml) was added to neutralise the trypsin or TrypLE. The harvested cell suspension was transferred to a sterile centrifuge tube and centrifuged at 1000 *g* for 5 min to pellet cells. The supernatant was discarded and cells pellet was resuspended in fresh complete medium. Cell density was

determined using a haemocytometer. Breast cancer cells were seeded out in cell culture flasks at a density of ~4 x 10^3 cells/cm^2 as recommended by LGC-ATCC standards, Teddington UK, while endothelial cells were seeded out at recommended density (10^3 cells/cm^2) by PromoCell, Heidelberg, Germany. Cells were incubated in the humidified incubator and growth medium was changed every 48 h.

2.3 Isolation of cancer cell-derived microparticles

The breast cancer cell lines MDA-MB-231, expressing high levels of tissue factor, and MCF-7 with low tissue factor expression were used throughout the study to obtain tissue factor-containing and tissue factor-deficient microparticles, respectively. Cells (~10^5 cells/well) were seeded in a 6-well culture plate and supplemented with reduced-serum medium (1 % v/v Glutamax, 5 % v/v FCS) (2 ml). To obtain cell-derived microparticles, medium was replaced with serum free medium and incubated for a further 3 h. The conditioned medium were then collected and centrifuged at 12,000 *g* for 20 min to pellet cell debris. The supernatant containing cell-derived microparticles was centrifuged at 100,000 *g* for 1 h at 20 °C to sediment the microparticles. Microparticle pellet were then resuspended in PBS (100 µl) and stored at -20 °C.

2.4 Analysis of microparticle density and microparticle-associated tissue factor antigen and activity

The density of microparticles isolated from MDA-MB-231 or MCF-7 breast cancer cells were determined using the Zymuphen microparticle assay kit (see section 2.4.1). Tissue factor content of microparticles was measured using a tissue factor enzyme-linked immunosorbent assay (ELISA) (see section 2.4.2). Microparticle-associated tissue factor activity was measured using a chromogenic assay based on thrombin generation (see section 2.4.3). In order to demonstrate that the thrombin generation

potential was derived from tissue factor, samples were pre-incubated with an inhibitory anti-tissue factor monoclonal antibody (25 µg/ml) for 30 min prior to measuring thrombin generation using the chromogenic assay.

2.4.1 Determination of microparticle density using the Zymuphen microparticle assay kit

Reagent R1 (bovine FXa-FVa mixture, containing calcium), reagent R2 (purified human prothrombin), reagent R3 (thrombin specific chromogenic substrate), microparticle calibrator and microparticle samples were thawed and equilibrated at 37 ºC before the experiment. Samples of isolated microparticles (20 µl) were placed in the provided 96-well microplate. To create the calibration curve, the microparticle calibrator was diluted to a range covering 0-2.1 nM and equal volumes (20 µl) were placed in a 96-well microplate. R1 reagent (50 µl) was then added to the wells, followed by R2 reagent (25 µl) and allowed to incubate for 10 min at 37 °C. R3 reagent (25 µl) was then added to all the test samples and allowed to develop for exactly 5 min at 37 °C in the dark. The reaction was then terminated by adding 2 % (v/v) citric acid (25 µl) and the absorption was measured at 405 nm using a plate reader. Microparticle densities were then determined from the calibration curve.

2.4.2 Determination of the tissue factor antigen by ELISA

To prepare the ELISA plate, sheep anti-human tissue factor-antibody was diluted 1:100 with coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) (100 µl) and placed in a 96-well microplate and then incubated overnight at 4 °C to coat the plate. Contents of the plate were then discarded and blocking buffer (PBS, pH 7.4, 1 % w/v BSA) (150 µl) was added to the wells and incubated for a minimum of 90 min at room temperature, or overnight at 4 °C. The plate was then washed four times with PBS-Tween buffer (PBS,

pH 7.4, 1 % v/v Tween 20) (200 µl). To prepare the standard curve, non-lipidated recombinant tissue factor was diluted with sample diluent (100 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), pH 7.2, 52 mM NaCl, 0.1 % v/v Triton X-100) to achieve final concentrations of 100, 50, 25 and 12.5 ng/ml. The tissue factor standards and samples (100 µl) were then aliquot into separate wells and incubated at 22 °C for 60 min. The plate was then washed four times with PBS-Tween buffer (200 µl) to remove any unbound tissue factor. The samples were probed with HRP-conjugated sheep anti-human tissue factor antibody diluted 1:400 with conjugated diluents (100 mM HEPES, pH 7.2, 52 mM NaCl, 1 % w/v BSA, 0.1 % v/v Triton X-100) (100 µl) and incubated for 60 min at room temperature. The plate was then washed four times with PBS-Tween buffer (200 µl) and developed by adding 3,3',5,5'-tetramethylbenzidine (TMB) substrate (100 µl) and incubated at room temperature for 15 min. The reaction was stopped using 2 M sulphuric acid (50 µl) and the absorption was measured at 490 nm using a plate reader. Tissue factor concentrations were then determined from the standard curve.

2.4.3 Analysis of tissue factor activity using chromogenic assay

Microparticle samples (20 µl) were placed into a 96-well plate along with the standard (20 µl). The standard curve was prepared from Innovin recombinant human tissue factor containing synthetic phospholipids, calcium, buffer and stabiliser, with the final concentrations of 1.6 ρM, 0.8 ρM, 0.4 ρM and 0.2 ρM (diluted with dH_2O). Both the standard and samples were then incubated with 0.5 M Tris-HCl buffer (pH 7.2) (30 µl), barium sulphate-adsorbed plasma (BSAP) (10 mg/ml) (30 µl), and calcium chloride (25 mM $CaCl_2$) (15 µl) for 30 min at 37 °C. The generation of thrombin was measured by the addition of substrate buffer (0.05 M Tris-HCl buffer, pH 7.2, 25 mM EDTA) (75 µl) containing 0.1 mM chromogenic substrate (N-Benzoyl-Phe-Val-Arg-p-nitroanilide

hydrochloride). Samples were incubated at 37 °C for a further 30 min. The reaction was stopped by the addition of 20 % (v/v) acetic acid (50 µl) and the absorption was measured at 405 nm using a plate reader. The tissue factor activity of the samples was determined from the standard curve.

2.5 Analysis of the rate of microparticle adhesion onto endothelial cells

HDBEC (~2 x 10^4 cells/well) were seeded in a 4-well glass chamber-slide and supplemented with complete medium (800 µl). On the following day, the medium was replaced with 1 % (v/v) reduced-serum medium (200 µl) and incubated at 37 ºC for a further 1 h prior to microparticles treatment. Microparticles were pre-labelled with fluorescent dye, 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI) (5 µM) for 20 min at room temperature. DiI is a fluorescent lipophilic dye that can diffuses laterally to stain the entire cell. DiI-labelled microparticles (0.3 nM) were added to HDBEC and incubated for up to 60 min at 37 ºC. Cells were then washed with sterile PBS (1 ml) to remove non-bound microparticles, fixed with formaldehyde (3 % v/v) for 30 min and then washed three times with PBS (1 ml). To prepare the microscope slide, the chambers were removed with a separation tool and then a cover slip was sealed to the glass bottom with nail varnish. Samples were then analysed using a fluorescence microscope with x20 objective.

2.6 Analysis of the transfer of tumour-derived microparticles to endothelial cells

In order to determine the binding of microparticles to the surface of the endothelial cells and the transfer of tissue factor, endothelial cells (~2 x 10^4 cells/well) were seeded in a

96-well microplate and supplemented with complete medium (200 µl). On the following day, the medium was replaced with reduced-serum medium (Human Endothelial Medium MV supplemented with 1 % v/v Endothelial Cell Growth supplement) (100 µl) and incubated for a further 1 h prior to incubation with microparticles. Microparticles derived from MDA-MB-231 or MCF-7 cells (0.3 nM) were added to the cells at 30 min intervals, up to 6 h and incubated at 37 °C. The medium was then removed and cells were washed one time with sterile PBS (100 µl) to remove any non-bound microparticles. Cell surface tissue factor antigen and tissue factor activity were then measured. To determine cell surface tissue factor antigen, cells were fixed with formaldehyde (3 % v/v) and then analysed using tissue factor ELISA (see section 2.4.2). To determine the cell surface tissue factor activity, cells were analysed immediately using a modified chromogenic assay (see section 2.4.3), using Tris-buffered saline (10 mM Tris-HCL, pH 7.0, 154 mM NaCl).

To examine cell surface tissue factor antigen by confocal microscopy, HDBEC (~5 x 10^4 cells/well) were seeded out into glass base dishes (35 mm µ-dishes) and supplemented with complete medium (2 ml). The medium was replaced with reduced-serum medium (100 µl) on the following day and incubated for a further 1 h prior to microparticles treatment. MDA-MB-231 or MCF-7 cell-derived microparticles (0.3 µm) were then added to HDBEC at intervals, up to 3 h. The medium was removed and HDBEC were washed one time with sterile PBS (1 ml) to remove non-bound microparticles. Cells were then fixed with 3 % (v/v) formaldehyde (200 µl) for 20 min, washed three times with PBS (2 ml) and then blocked with donkey serum (5 % v/v) for 30 min. Tissue factor surface antigen was labelled with an FITC-conjugated anti-human tissue factor (diluted 1:100) for 2 h. Cells were washed three times with PBS (2 ml) to

remove non-bound antibody and images were acquired using Zeiss LSM 710 confocal microscope with x63 water immersion objective and ZEN software.

2.7 Analysis of the internalisation of tumour-derived microparticles by endothelial cells

HDBEC (~5 x 10^4 cells/well) were seeded out in glass base dishes (35 mm µ-dishes) and supplemented with complete medium (2 ml). The medium was replaced with 1 % (v/v) reduced-serum medium (100 µl) on the following day and incubated for a further 1 h. In order to examine the involvement of endocytosis in the internalisation of tissue factor–bearing microparticles, HDBEC were pre-incubated with a dynamin inhibitor, Dynasore (3-Hydrocy-naphthalene-2-2carboxylic acid (3,4-dihydroxyl-benzylidene)-hydrazide hydrate, 15 µM) for 30 min prior to incubation with microparticles. The set of cells was incubated with tissue-factor bearing microparticles for 2.5 h and washed one time with PBS (2 ml) to remove any non-bound microparticles. The cells were then fixed with 3 % (v/v) formaldehyde (200 µl) for 30 min, washed three times with PBS (2 ml) and then blocked with donkey serum (5 % v/v) for 30 min. Cell surface tissue factor was labelled with an FITC-conjugated anti-tissue factor antibody (diluted 1:100) for 2 h at room temperature and then washed three times with PBS (2 ml). Images were acquired using Zeiss LSM 710 confocal microscope with x63 water immersion objective and ZEN software.

2.8 Analysis of tissue factor mRNA expression

HDBEC (~10^6 cells/well) were seeded in a 24-well plate and supplemented with complete medium (2 ml). On the following day, medium was replaced with 2 % (v/v) reduced-serum medium (2 ml) and incubated for 2 h at 37 ºC. Microparticles derived from MDA-MB-231 or MCF-7 cells (0.3 nM) were added to the cells at intervals, up to

24 h. Medium was then removed and cells were washed one time with PBS (2 ml). Total RNA was isolated from cells using TRI-reagent system (see section 2.8.1) and quantified by Real-time polymerase chain reaction (RT-PCR) (see section 2.8.3)

2.8.1 RNA Isolation by TRI Reagent

Following treatment with microparticles, cells were harvested using TrypLE (see section 2.2.3) and pelleted by centrifuging at 12,000 *g* for 15 min. Cell pellet was then lysed using TRI reagent (200 µl) by repeated pipetting and the homogenate was allowed to stand for 5 min at room temperature. To separate RNA from DNA and proteins, chloroform (40 µl) was added to the samples, vortexed for 15 s and left to stand at room temperature for 15 min. Samples were then centrifuged at 12,000 rpm for 15 min to separate the mixture into three phases: a lower red phenol-chloroform phase (containing proteins and small fragments of DNA), an interphase (containing large fragment of DNA and proteins), and the colourless upper aqueous phase (containing RNA). The RNA-containing colourless aqueous phase was then transferred to a fresh RNase-free tube and mixed with isopropanol (100 µl) by gently inverting. The samples were allowed to stand for 60 min at -20 °C and then centrifuged at 12,000 rpm to pellet the RNA. The supernatant was removed and RNA pellet was washed with 75 % (v/v) ethanol (200 µl) by vortexing, followed by centrifugation at 12,000 rpm for 5 min. Ethanol was removed and the pellet was resuspended in RNase-free water (60 µl). The samples were stored at -70 °C until required.

2.8.2 Quantification of the concentration of RNA

The concentration of RNA was determined using a spectrophotometer by measuring the absorption of the test samples (6 µl) diluted in RNase-free water (54 µl) at 260 nm in a quartz cuvette calibrated against a water blank. An A_{260} reading of 1.0 is equivalent to

40 µg/ml single-stranded RNA, therefore the concentration of the RNA sample was calculated using the following equation:

$$\text{RNA concentration (µg/ml)} = A_{260} \times 40 \text{ µg/ml} \times \text{dilution factor}$$

2.8.3 Quantification of tissue factor mRNA expression by RT-PCR

SYBR ® Green RT-PCR mix (2X), RT enzyme mix (125X), primer (400 nM) and RNA template (100 ng) were thawed prior to the experiment and kept on ice. The RT-PCR master mix was prepared on ice, in a microcentrifuge tube according to Table 2.1 and mixed well. The master mix was pipetted into a 96-well PCR plate. The plate was then sealed with optical adhesive film and RT-PCR was carried out using an iCycler thermal cycler according to the cycling conditions shown in Table 2.2.

A melt curve was also performed to detect non-specific amplification. The mRNA levels of tissue factor were normalised against the mRNA levels of a housekeeping gene, β-actin. The sequences of tissue factor and β-actin primers for RT-PCR were:

Tissue factor-forward: 5'-ACC TGG AGA CAA ACC TCG GAC-3'

Tissue factor-reverse: 5'-GAG TTC TCC TTC CAG CTC TGC-3'

β-actin-forward: 5'-TGATGGTGGGCATGGGTCAGA-3'

β-actin-reverse: 5'-GTCGTCCCAGTTGGTGACGAT-3'

Table 2.1: RT-PCR master mix

Reagent	Volume for 1 reaction (µl)	Final Concentration
Power SYBR® Green RT-PCR Mix (2X)	10 µl	1X
Forward Primer	Variable	200 nM
Reverse Primer	Variable	200 nM
RT Enzyme Mix (125X)	0.16 µl	1X
RNA template	Variable	100 ng
RNase-free H_2O	Variable	-
Total	**20 µl**	-

Table 2.2: The cycling conditions of RT-PCR

Stage	Step	Temperature (°C)	Time
Holding	Reverse transcription	48	30 min
Holding	Activation of AmpliTaq Gold® RNA polymerase Ultra Pure	95	10 min
Cycling (40 cycles)	Denature	95	15 s
	Anneal/ Extend	60	1 min
Melt Curve	Denature	95	15 s
	Anneal	60	15 s
	Denature	95	15 s

2.9 Analysis of the exposure of phosphatidylserine on the cell surface

HDBEC (~5 x 10^4 cells/well) were seeded out into glass base dishes (35 mm µ-dishes) and supplemented with complete medium (2 ml). The medium was replaced with 1 % (v/v) reduced-serum medium (100 µl) on the following day and incubated for a further 1

h prior to incubation with microparticles. HDBEC were incubated with tissue factor-bearing microparticles derived from MDA-MB-231 cells for up to 2 h. At various intervals, sample of cells were washed one time with HEPES buffer (25 mM HEPES, pH 7.2, 150 mM $NaCl_2$, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$) and then fixed with formaldehyde (3 % v/v) for 30 min. The cells were then labelled with Alexa Fluor 647-labelled annexin V (diluted 1:200) and incubated at room temperature for 15 min. The cells were washed three times with HEPES buffer and analysed using a Zeiss LSM 710 confocal microscope with x63 water immersion objective and ZEN software.

2.10 Cloning of recombinant tissue factor into pEGFP-C3 plasmid

2.10.1 Purifications of plasmid DNA using Wizard Miniprep purification system

TB-1 *E.*coli containing pEGFP-C3 (200 µl) was cultured in Luria Broth medium (1 % w/v Tryptone, 1 % w/v NaCl, 0.5 % w/v yeast extract) (50 ml) and incubated overnight at 37 °C with shaking at 170 rpm. On the following day, TB1 culture was centrifuged at 2500 *g* for 30 min to pellet cells. The cell pellet was then resuspended in cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/ml RNase A) (3 ml). Lysis Solution (0.2 M NaOH, 1 % v/v SDS) (3 ml) was mixed by inverting and left at room temperature for 2 min to allow full lysis. The lysis solution was neutralised by the addition of neutralisation solution (1.32 M potassium acetate, pH 4.8) (3 ml). The lysate was then centrifuged at 3000 *g* for 20 min at 4 °C. In order to isolate the plasmid DNA, resuspended DNA-binding resin (10 ml) was added to the DNA-containing supernatant in a 15 ml midicolumn and attached to a vacuum minifold. A vacuum was then applied and released when all liquid has passed through the column. A column wash solution (80 mM potassium acetate, 8.3 mM Tris-HCl, pH 7.5, 40 µM EDTA, 55 % v/v ethanol) (8 ml) was then added and vacuum was applied to pull the liquid through column. When all the liquid has passed through the column, the vacuum was release

and the midicolumn was separated from the reservoir. The midicolumn was then placed in a 1.5 ml microcentrifuge tube and centrifuged at 13,400 rpm for 5 min to remove the residue of wash solution. To elute plasmid DNA, the midicolumn was transferred to a new 1.5 ml microcentrifuge tube, followed by the addition of preheated water (60 °C) (300 µl) and left to stand at room temperature for 1 min, and then centrifuged at 13,400 rpm for 1 min. Samples were stored at -20 °C until required. The plasmid DNA was examined by TBE agarose gel electrophoresis (see section 2.10.2) and the concentration of plasmid DNA was quantified using a UV spectrophotometer (see section 2.10.3).

2.10.2 Separation of DNA using agarose gel electrophoresis

Agarose (0.25 g) was dissolved in Tris-borate EDTA (TBE) electrophoresis buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.3) (50 ml) in a conical flask to a final concentration of 0.5 % (w/v) by heating in a microwave oven. Once completely dissolved, the agarose solution was poured into a sealed electrophoresis tray with an appropriate comb in place. The gel was left to set at room temperature for 15 min and then placed at 4 ºC for 20 min. When the gel had set, the comb was removed and the gel was placed into the electrophoresis tank containing enough TBE electrophoresis buffer (1 X) to cover the gel. A 1 kb DNA ladder (5 µl) was mixed with SYBR Green nucleic acid I gel stain (1 µl) and loaded into one of the wells. The test samples (10 µl) were mixed with SYBR Green nucleic acid 1 gel stain (1 µl) and 6X blue loading dye solution (0.1 % w/v bromophenol blue, 30 % w/v glycerol) (5 µl) and loaded into separate wells. Electrophoresis was carried out at 100 V for approximately 45 min until the tracking dye was approximately 2 cm away from the end of the gel. The gel was visualised under UV light using 3UV^{TM} transilluminator and images were taken using GeneSnap image capture system.

2.10.3 Determination of the concentration of plasmid DNA

The concentration of total DNA was determined using a spectrophotometer by measuring the absorption of test samples (6 µl) diluted in DNase-free water (54 µl) at 260 nm in a quartz cuvette calibrated against a water blank. An A_{260} reading of 1.0 is equivalent to 50 µg/ml single-stranded DNA, therefore the concentration of plasmid DNA was calculated using the following equation:

$$\text{DNA concentration (µg/ml)} = A_{260} \times 50 \text{ µg/ml} \times \text{dilution factor}$$

2.10.4 Polymerase chain reaction (PCR) of tissue factor cDNA using platinum Taq DNA polymerase high fidelity

Tissue factor cDNA of 789 bp was cloned by PCR from a pCMV6-XL5-TF plasmid containing the full length DNA sequence of tissue factor. The PCR master mix was prepared in a PCR tube according to Table 2.3 and mixed well. The PCR was carried out for 25 cycles, following the cycling conditions shown in Table 2.4. The sequences of tissue factor primers used in the PCR were:

Tissue factor-forward: 5'-GCTCAAGCTTTCAGGCACTACAAA-3'

Tissue factor-reverse: 5'-CGGTGGATCCTTATGAAACATTCA-3'

The primer annealing temperature was determined based on the primer melting temperature (Tm), generally about 5°C lower than the melting temperature. Primer melting temperature was calculated based on the following formula:

4(G+C) + 2 (A+T) °C.

Tm of tissue factor-forward = 4(G+C) + 2 (A+T) °C

= 4 (4+7) + 2 (8+5)

= 70 °C

Tm of tissue factor-reverse = 4 (G+C) + 2 (A+T) °C

= 4 (5+5) + 2 (7+7)

= 68 °C

The PCR products were then examined using agarose gel (1.5 % w/v) electrophoresis (see section 2.10.2).

Table 2.3: PCR master mix

Reagents	**Volume for 1 reaction (µl)**	**Final Concentration**
DNA Template (pCMV-XL5- TF)	2	150 ng
10 µM Primer Mix	2	0.2 µM
10X High Fidelity Buffer	10	1X
10 mM dNTP mixture	2	0.2 µM
Platinum® *Taq* DNA polymerase	0.4	1 unit
50 mM $MgCl_2$	4	2 mM
dH_20	79.6	-
Total	**100**	**-**

Table 2.4: The cycling conditions of PCR

Stage	Temperature (°C)	Time	Number of Cycles
Initial denaturation	94	2 min	1
Denaturation	94	30 s	25
Annealing	64	30 s	
Extension	68	1 min	
Final extension	68	10 min	1

2.10.5 Digestion of DNA using restriction enzymes

Samples for DNA digestion were prepared in a 0.2 ml tubes respectively according to Table 2.5. To activate the restriction enzymes, samples were incubated at 37 °C and reaction was carried out for 3.5 h for complete DNA digestion.

Table 2.5: The master mix for DNA digestion

Reagent	Volume (µl)	Final Concentration
DNA (pEGFP-C3 or tissue factor)	20	1 µg
10X Buffer	3	1X
10X diluted BSA	3	1X
Bam HI (10 U/ml)	1	0.3 U/ml
Hind III (10 U/ml)	1	0.3 U/ml
dH_20	2	-
Total	**30**	-

2.10.6 DNA purifications from enzymatic reactions

Following DNA digestion, pEGFP-C3 and tissue factor DNA were purified off the enzymatic reaction using GFX^{TM} PCR DNA and Gel Band purification system. The GFX column was placed into a collection tube. Capture buffer 2 (buffer solution containing acetate and chaotrope) (500 µl) was added to the samples (30 µl), mixed thoroughly and transferred onto the assembled GFX-collection tube. The assembled column-collection tube was then centrifuged at 13,400 rpm for 1 min and the flow through was discarded by emptying the collection tube. Wash buffer type 1 (Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 80 % v/v ethanol) (500 µl) was then added to the column and centrifuged at 13,400 rpm for 1 min. The column was transferred to a fresh 1.5 ml DNase-free microcentrifuge tube. To elute DNA, elution buffer type 4 (10 mM Tris-HCl, pH 8.0) (50 µl) was added to the centre of the membrane in the column, incubated at room temperature for 1 min, and then centrifuged at 13,400 rpm for 1 min. Samples were stored at -20 °C until required.

2.10.7 T4 DNA Ligation

Following DNA purification from enzymatic reactions, the concentration of digested plasmid pEGFP-C3 and tissue factor DNA were determined by measuring the absorption at 260 nm using UV spectrophotometer and calculated using the following equation:

$$\text{Concentration of plasmid (pmol/ul)} = \frac{\text{DNA concentration of pEGFP-C3/ insert TF (ug/ml)} \times 10^6}{660 \times \text{size of plasmid} \times 1000}$$

DNA ligation was set up in 0.2 ml tubes at the ratio 1:3, 1:5 of plasmid insert. A negative control (without insert-tissue factor) was included. Samples were prepared according to Table 2.6 and incubated overnight at 15 °C.

Table 2.6: The master mix for DNA Ligation

Reagent	**Ratio**		
	1:3	**1:5**	**Negative Control**
5X Ligase Reaction Buffer	4	4	4
Vector DNA (pEGFP-C3)	3 to 30 f-mol	3 to 30 f-mol	3 to 30 f-mol
Insert DNA (Tissue factor)	9 to 90 f-mol	15 to 150 f-mol	-
T4 DNA Ligase (U)	1 U/ml	1 U/ml	1 U/ml
Rnase-free water	Variable	Variable	Variable
Total	**20**	**20**	**20**

2.10.8 Bacteria transformation

After DNA ligation, competent *E.*coli TB1 bacteria were transformed using the recombinant plasmid DNA. DMSO (3 µl) was added to the competent TB1 (200 µl) and mixed briefly. Recombinant plasmid DNA (100 ng) was then added to competent TB1 (100 µl) and incubated on ice for 30 min. To introduce the plasmid DNA into competent TB1, cells were heat shocked at 42 ºC for 2 min and then cooled down to 4 ºC for 1 min. SOC media (Tryptone 2 % (w/v), Yeast extract 0.5 % (w/v), 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20 mM Glucose) (100 µl) was then added to the cells and incubated for 1 h at 37 ºC. Cells (75 µl) was then plated out on the Kanamycin (50 µg/ml) Luria Broth agar plate and incubated overnight at 37 ºC.

Chapter 3

Results

3.1 Determination of the time point for the maximal release rate of microparticles from cancer cells, following protease activated receptor 2 (PAR2) activation

In order to determine the optimal time point to achieve maximal release of microparticles, MDA-MB-231 and MCF-7 cells were treated with PAR2-agonist peptide (PAR2-AP) (20 μM) for 45 or 90 min and the microparticles were isolated from the conditioned medium. The concentration of microparticles in each sample was determined using the Zymuphen microparticle assay kit as described in section 2.4.1. In addition, the concentration of tissue factor antigen within each sample was measured using a tissue factor antigen ELISA as described in section 2.4.2. High rates of microparticle release from MDA-MB-231 and MCF-7 cells were detected at 45 min post-stimulation with PAR2-AP (Figure 3.1A & Figure 3.2A). In addition, the highest levels of tissue factor release were detected at 45 min post-stimulation in MCF-7 cells. However, no significant amount of tissue factor was detected in microparticles isolated from non-activated MCF-7 cells, suggesting that PAR2 activation in MCF-7 resulted in the release of tissue factor into microparticles (Figure 3.2B). In contrast, high levels of tissue factor (ranging from 91-176 ng/ml) were detected in all microparticle samples derived from MDA-MB-231 cells, including non-activated MDA-MB-231, indicating that MDA-MB-231 cells constitutively release tissue factor into microparticles (Figure 3.1 B). However, because microparticles are readily taken up by cells, it has to be noted that the decrease in tissue factor concentration in the cell culture medium of MCF-7 and MDA-MB-231 observed at 90 min represents the net flux of tissue factor-bearing microparticles (Figure 3.1 & Figure 3.2).

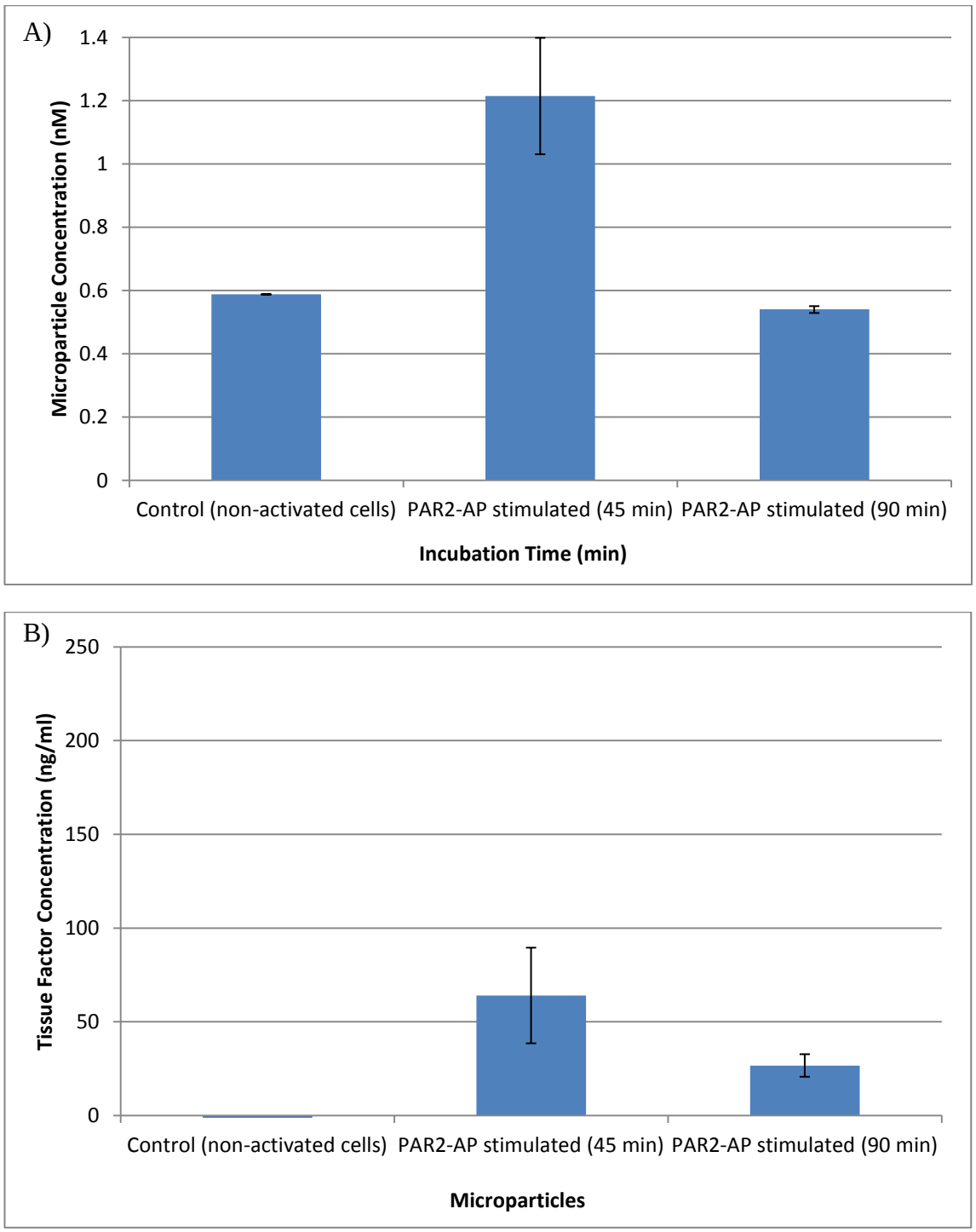

Figure 3.1: Analysis of the release of microparticles from MCF-7 cells.

MCF-7 cells (~10^5 cells/ well) were seeded in a 6-well plate and adapted to serum free medium. Cells were activated by adding PAR2-AP (20 µM) for 45 and 90 min. A non-activated control sample was included. Microparticles were then isolated from the conditioned medium of the samples. (A) Microparticle densities were determined by Zymuphen microparticle determination kit (n=6) (mean SE±0.03) and (B) the concentration of total tissue factor antigen of microparticles was determined by ELISA (n=3) (mean SE±1.73).

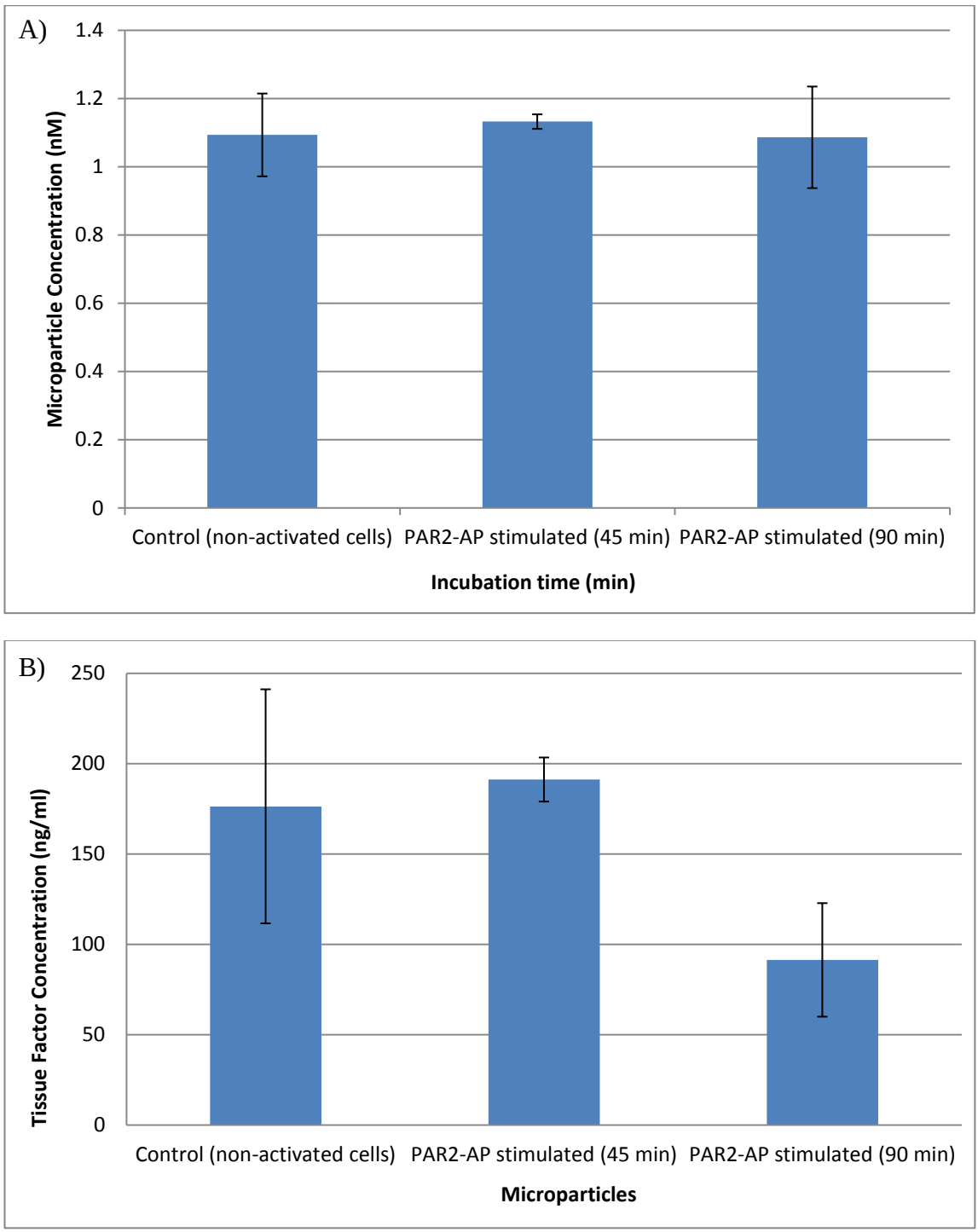

Figure 3.2: Analysis of the release of microparticles from MDA-MB-231 cells.

MDA-MB-231 cells (~10^5 cells/ well) were seeded in a 6-well plate and adapted to serum free medium. Cells were activated by adding PAR2-AP (20 µM) for 45 and 90 min. A non-activated control sample was included. Microparticles were then isolated from the conditioned medium of the samples. (A) Microparticle densities were determined by Zymuphen microparticle determination kit (n=6) (mean SE±0.04) and (B) the concentration of total tissue factor antigen of microparticles was determined by ELISA (n=3) (mean SE±10.8).

3.2 Analysis of the tissue factor-dependent procoagulant activity in cancer-derived microparticles

Tissue factor-bearing microparticles were isolated from conditioned medium obtained from non-activated MDA-MB-231 and MCF-7 cells by ultracentrifugation. The microparticle-associated tissue factor activity was determined by measuring thrombin generation, using a 2-stage chromogenic assay as described in section 2.4.3. Tissue factor activity in MDA-MB-231-derived microparticles was determined to be 107 U/ml, measured against a standard prepared using Innovin recombinant human tissue factor (Figure 3.3). Incubation of these MDA-MB-231-derived microparticles with an inhibitory anti-tissue factor antibody (25 µg/ml) suppressed the thrombin generation within these microparticles by more than 80 % (Figure 3.3) suggesting that the chromogenic activity associated with these microparticles was primarily derived from tissue factor. In contrast, no significant tissue factor activity was detected on microparticles obtained from MCF-7 cells (data not shown).

3.3 Analysis of the rate of microparticle adhesion onto endothelial cells

In order to measure the rate of microparticle adhesion onto the cell surface of endothelial cell, microparticles were pre-labelled with DiI prior to incubation with HDBEC. HDBEC was then treated with the DiI labelled-microparticles and microparticle adhesion was examined using fluorescence microscopy over 60 min. Incubation of HDBEC with DiI-labelled microparticles resulted in increased cell surface fluorescence within 5 min and peaked at 30 min, suggesting the rapid adherence and accumulation of microparticles to the cell surface of HDBEC (Figure 3.4). Furthermore,

no significant cell surface florescence was detected on HDBEC treated with microparticles for 45 and 60 min (Figure 3.4).

3.4 Analysis of the transfer of tissue factor from tumour-derived microparticles to microvascular endothelial cells

In order to examine the possibility of the transfer of tissue factor from tumour derived-microparticles to endothelial cells, HDBEC was incubated with tissue factor-bearing microparticles (0.3 nM) isolated from MDA-MB-231 cell line, or tissue factor-deficient microparticles (0.3 nM) isolated from MCF-7 cell line, up to 360 min. The surface tissue factor antigen and tissue factor activity of HDBEC were then measured using a tissue factor antigen ELISA and a 2-stage chromogenic assay, respectively (section 2.4.2 and 2.4.3). A sharp increase in surface tissue factor antigen was observed within 30 min on incubation of HDBEC with tissue factor-bearing microparticles but rapidly decreased to lower values between 60-90 min. This was followed by a second prolonged peak of tissue factor reappearance on the cell surface at 120 min, which lasted for a further 240 min (Figure 3.5A). However, the concentrations of cell surface tissue factor antigen were not concurrent with the tissue factor activity measured on the HDBEC; there was only a modest increase in tissue factor activity at 30 min which corresponded to the first tissue factor antigen peak. In contrast, high levels of tissue factor activity were detected at 120 min (Figure 3.5B). In comparison, no significant amount of surface tissue factor antigen (Figure 3.6A) or tissue factor activity (Figure 3.6B) were detected on HDBEC incubated with tissue factor-deficient microparticles derived from MCF-7 cell line.

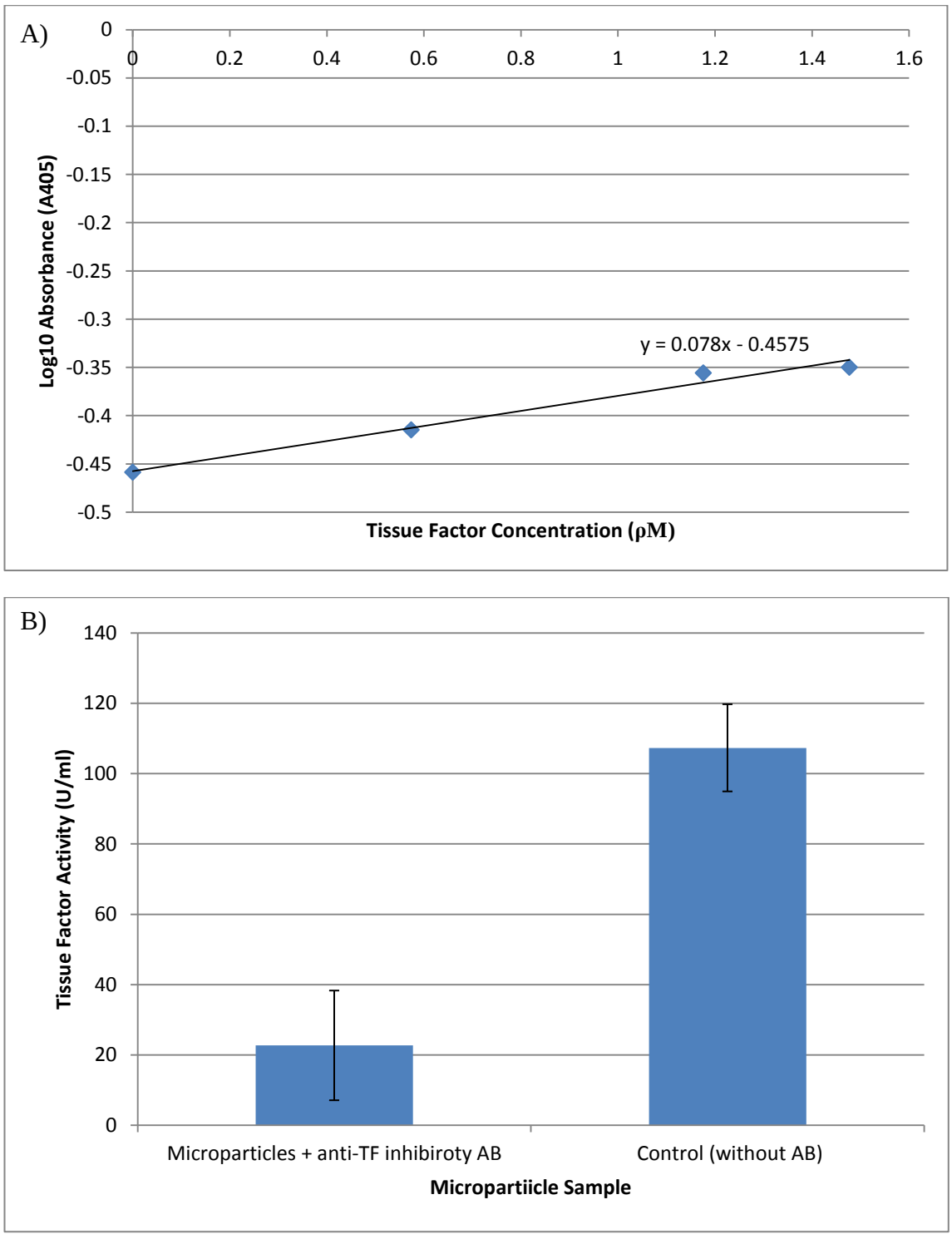

Figure 3.3: Analysis of the tissue factor activity within MDA-MB-231 derived-microparticles.

MDA-MB-231 tissue factor-bearing microparticles were pre-treated with 25 µg/ml anti-tissue factor inhibitory antibody. A control sample (without antibody) was included. (A) A standard curve was prepared using Innovin recombinant tissue factor and (B) the tissue factor activity in each sample was measured by a chromogenic assay, calculated against the standard curve (n=3) (mean SE±5). Ab: Antibody

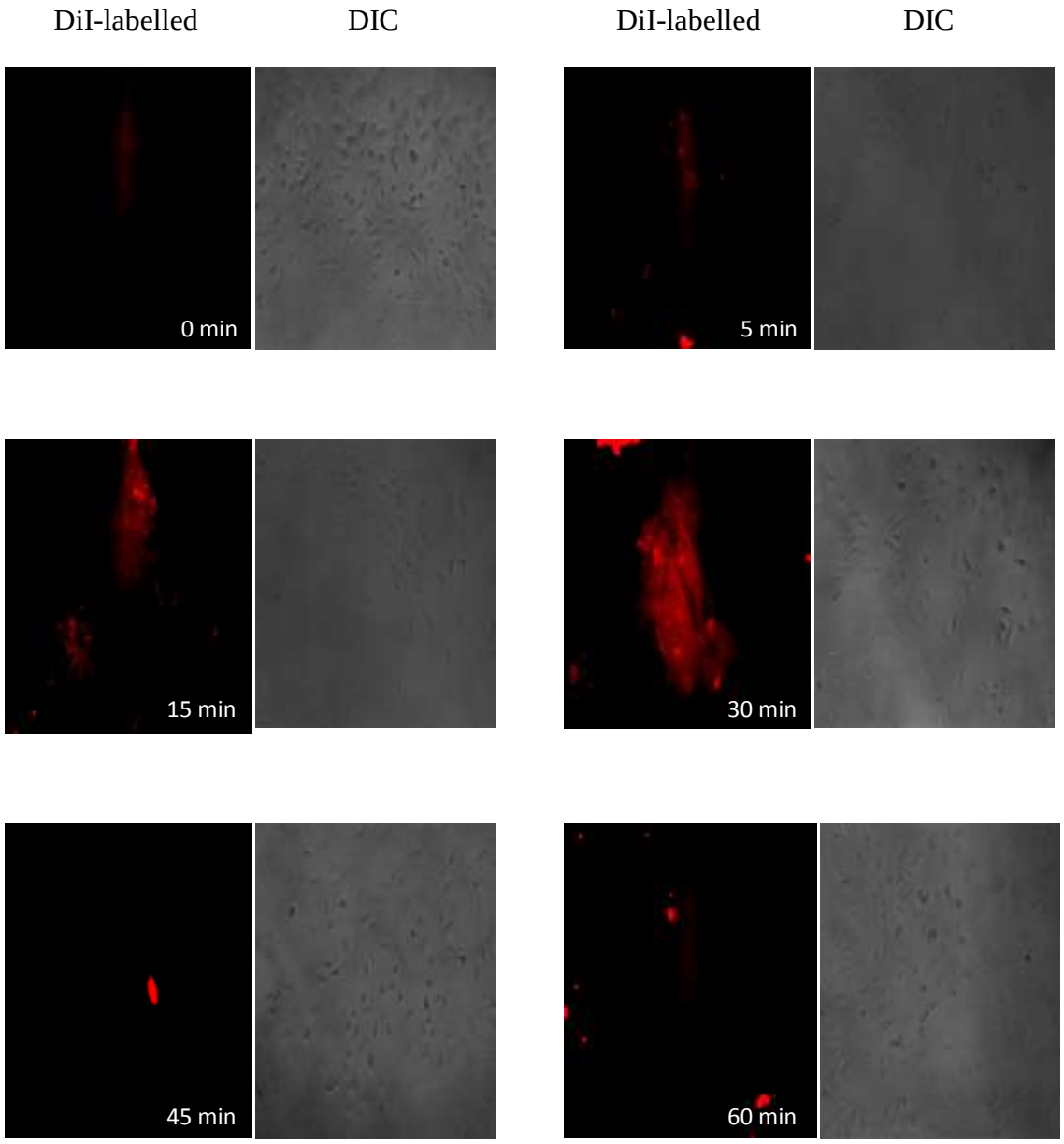

Figure 3.4: Analysis of the adherence of microparticles to endothelial cells by fluorescence microscopy.

HDBEC were incubated with DiI-labelled tissue factor-bearing microparticles derived from MDA-MB-231 cells for up to 60 min. The cells were then fixed with 3 % formaldehyde, washed with PBS, and analysed by fluorescence microscopy. Differential interference contrast (DIC) microscopic images are shown alongside (magnification x20).

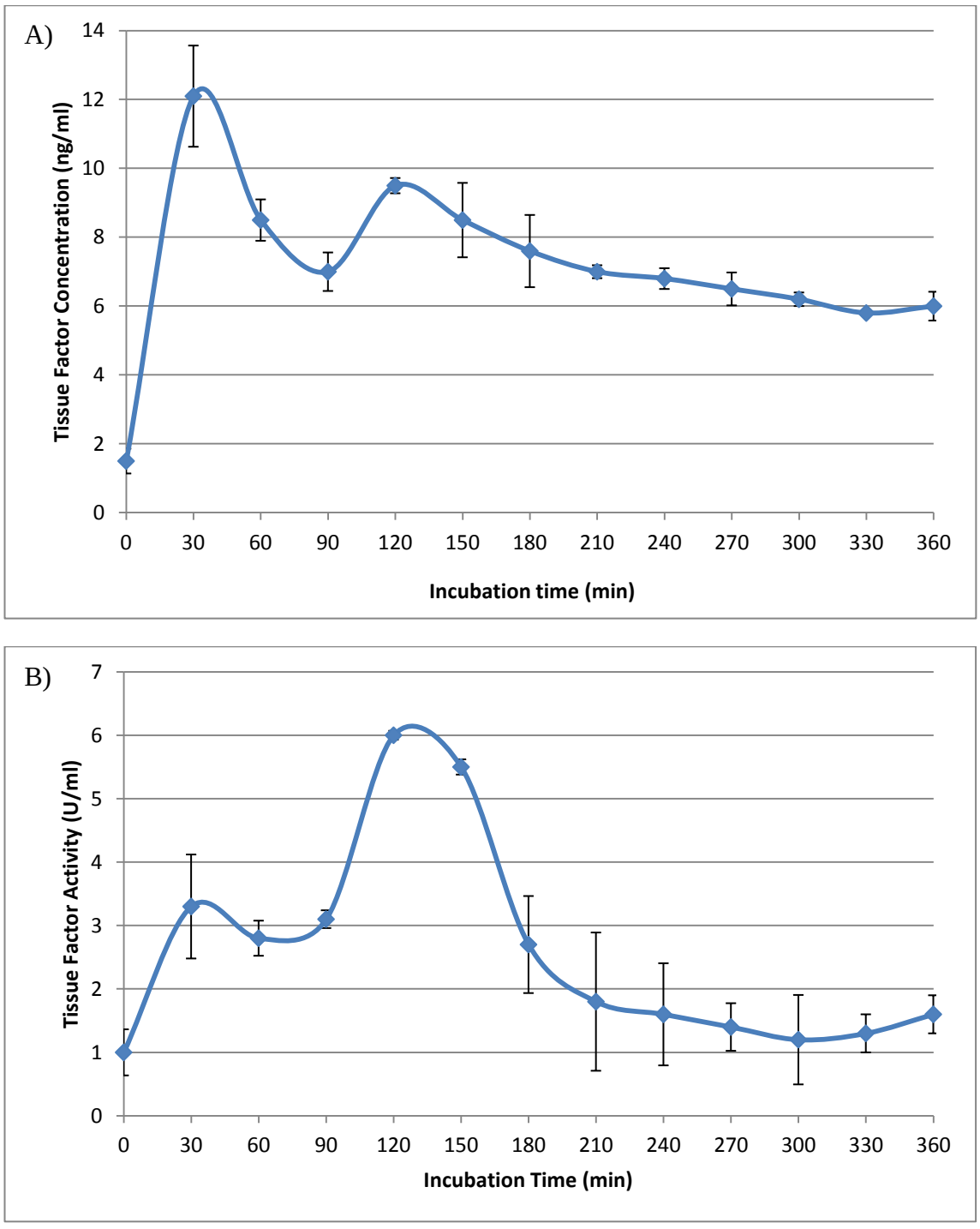

Figure 3.5: Time-course analysis of the transfer of tissue factor from MDA-MB-231 derived microparticles to microvascular.

HDBEC were incubated with tissue factor-bearing microparticles derived from MDA-MB-231 cells for up to 6 h following which, the cells were washed and fixed. (A) The concentration of surface tissue factor antigen was determined by ELISA (n=4), $p>0.05$ (mean SE±0.148) and (B) the tissue factor activity of HDBEC was determined using a chromogenic assay (n=3), $p>0.05$ (mean SE±0.13).

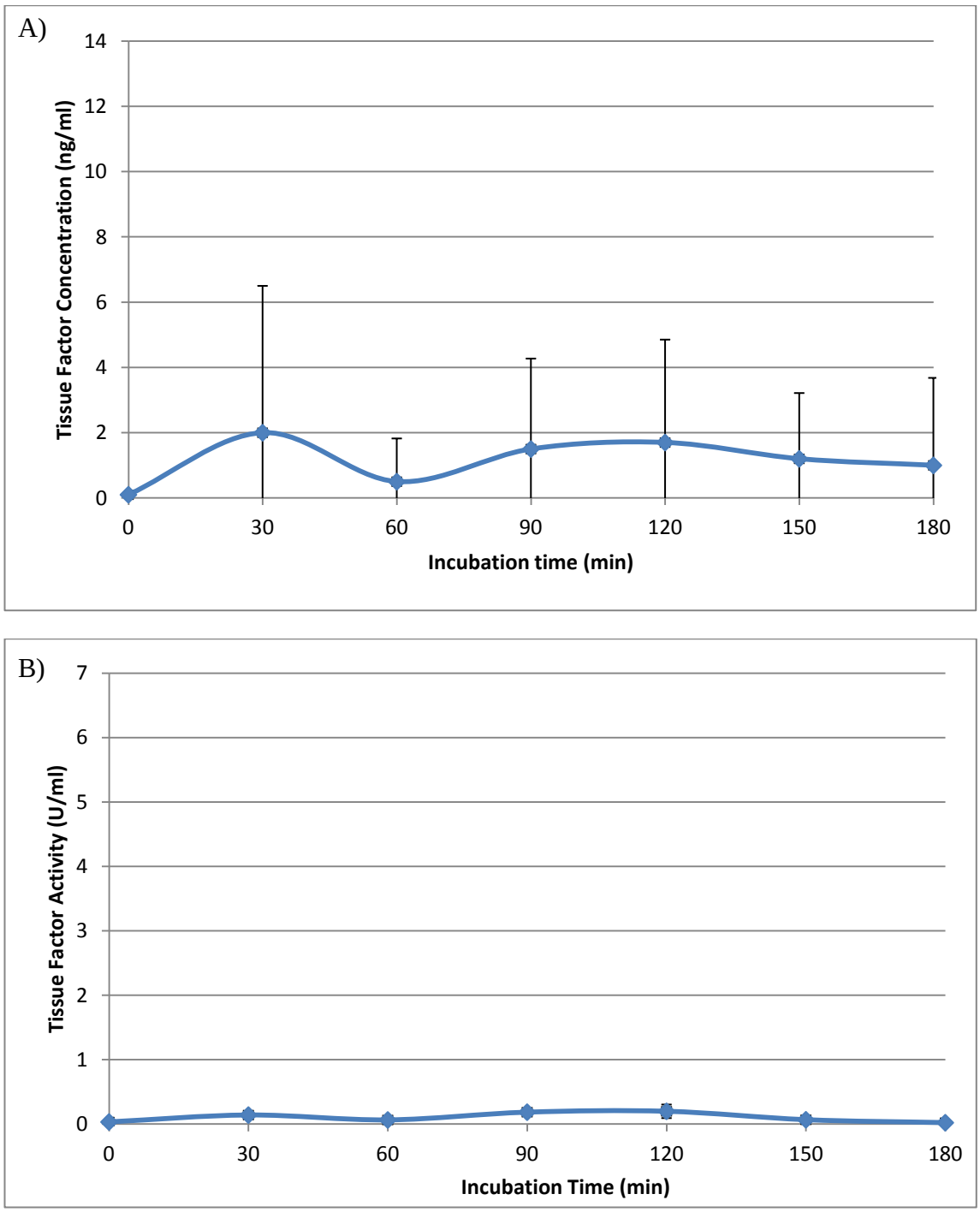

Figure 3.6: Time-course analysis of the transfer of tissue factor from MCF-7 to microvascular cells.

HDBEC were incubated with tissue factor-deficient microparticles derived from MCF-7 cells for up to 3 h following which, the cells were washed and fixed. (A) The concentration of surface tissue factor antigen was determined by ELISA (n=4), $p>0.05$ (mean±1.03) and (B) the tissue factor activity of HDBEC was determined using a chromogenic assay (n=3), $p>0.05$ (mean SE±0.013).

The transfer of tissue factor was also analysed by confocal microscopy at intervals up to 2 h, using an FITC-conjugated anti-tissue factor antibody. Analysis of surface tissue factor by confocal microscopy also showed the presence of tissue factor on the surface of endothelial cells at 30 and 120 min, but not at 60 min following incubation with MDA-MB-231 derived microparticles (Figure 3.7). In contrast, no detectable fluorescence was observed on HDBEC on incubation with tissue factor-deficient microparticles isolated from MCF-7 cells (Figure 3.7), or on untreated cells (Figure 3.7).

3.5 Analysis of the exposure of phosphatidylserine on the cell surface of endothelial cells following the incubation with microparticles

In order to examine the likelihood of exposure of phosphatidylserine on the cell surface of endothelial cells, HDBEC were incubated with tissue factor-bearing microparticles and were examined at intervals up to 120 min by labelling with Alexa Fluor 647-labelled annexin V. Labelling of HDBEC with fluorescent-labelled annexin V following incubation with microparticles isolated from the medium of MDA-MB-231 cells resulted in the formation of discrete patches containing phosphatidylserine at 30 min which then disappeared by 60 min incubation. Furthermore, diffuse phosphatidylserine-labelling was detected on the cell surface at 90 min post incubation and significantly increased by 120 min post-incubation (Figure 3.8).

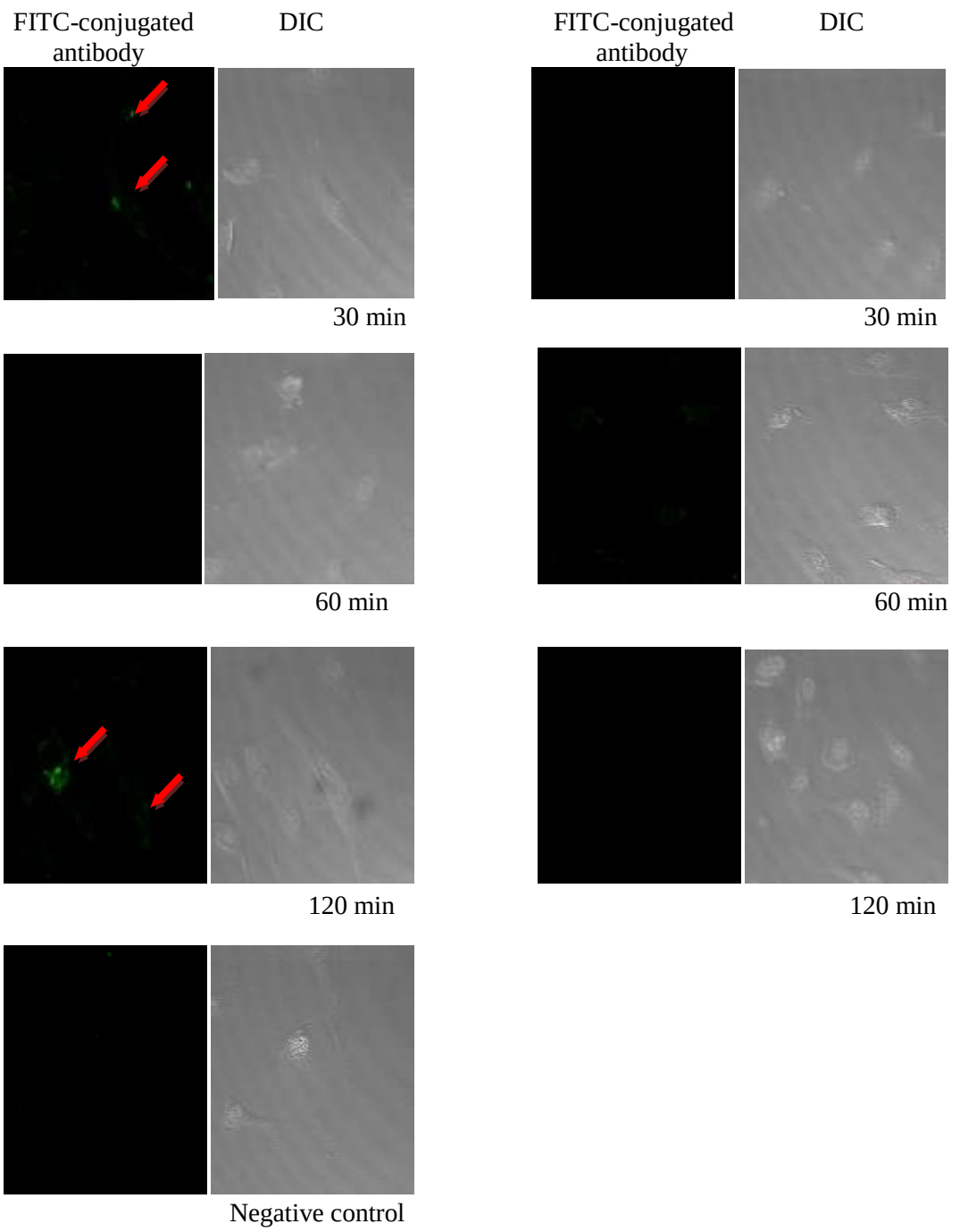

Figure 3.7: Analysis of the transfer of tissue factor from tumour-derived microparticles to endothelial cells by confocal microscopy.

HDBEC were incubated with tissue factor-bearing or tissue factor-deficient microparticles up to 2 h, following which the cells were fixed with 3 % formaldehyde, washed with PBS. A negative control (without microparticles) was included. Cell surface tissue factor was examined by labelling with an FITC-conjugated tissue factor antibody and analysed by confocal microscopy. Differential interference contrast (DIC) microscopic images are shown alongside (magnification x63).

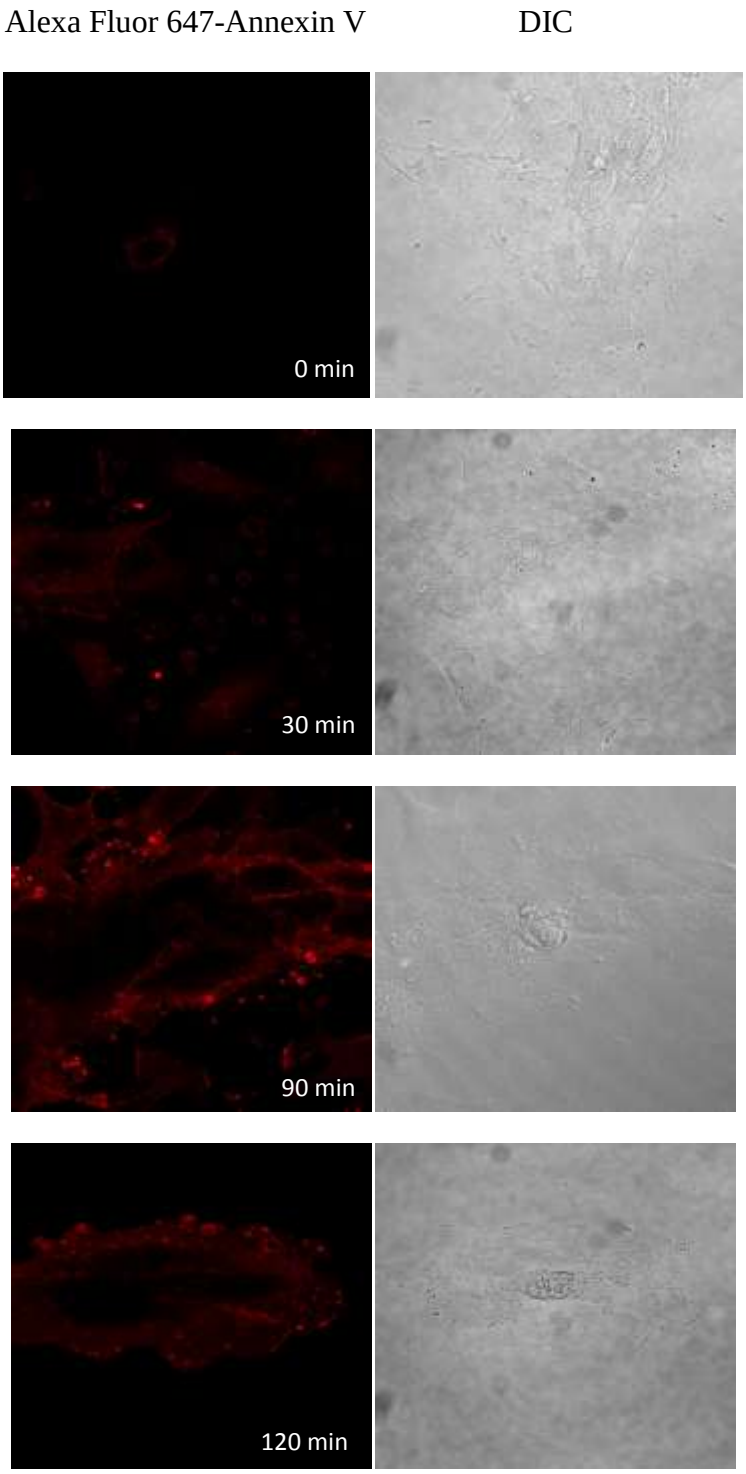

Figure 3.8: Analysis of the exposure of phosphatidylserine on the cell surface of endothelial cells by confocal microscopy.

HDBEC were incubated with tissue factor-bearing microparticles for up to 120 min following which, the cells were washed with HEPES buffer and fixed with 3 % formaldehyde. Surface phosphatidylserine was examined by labelling with Alexa Fluor 647-labelled annexin V and subsequently analysed with confocal microscopy. Differential interference contrast (DIC) microscopic images are shown alongside (magnification x63).

3.6 Analysis of the up-regulation of tissue factor expression in microvascular cells in response to tumour-derived microparticles

In order to determine if tissue factor expression within HDBEC was up-regulated in response to tumour-derived microparticles, HDBEC were incubated with tissue factor-bearing or tissue factor-deficient microparticles and the levels of tissue factor mRNA were examined at intervals up to 24 h. Total RNA was extracted from HDBEC as described in section 2.8.1 and the amount of tissue factor mRNA was determined using quantitative real-time RT-PCR. Analysis of the tissue factor mRNA expression in HDBEC, following incubation with either tissue factor-bearing or tissue factor-deficient microparticles showed no significant amount of tissue factor mRNA (<0.3 ng/ml) was detected. In addition, result showed no significant alteration in tissue factor mRNA expression at any time point, comparing to control sample, discounting the *de novo* expression of tissue factor in response to tumour-derived microparticles (Figure 3.9).

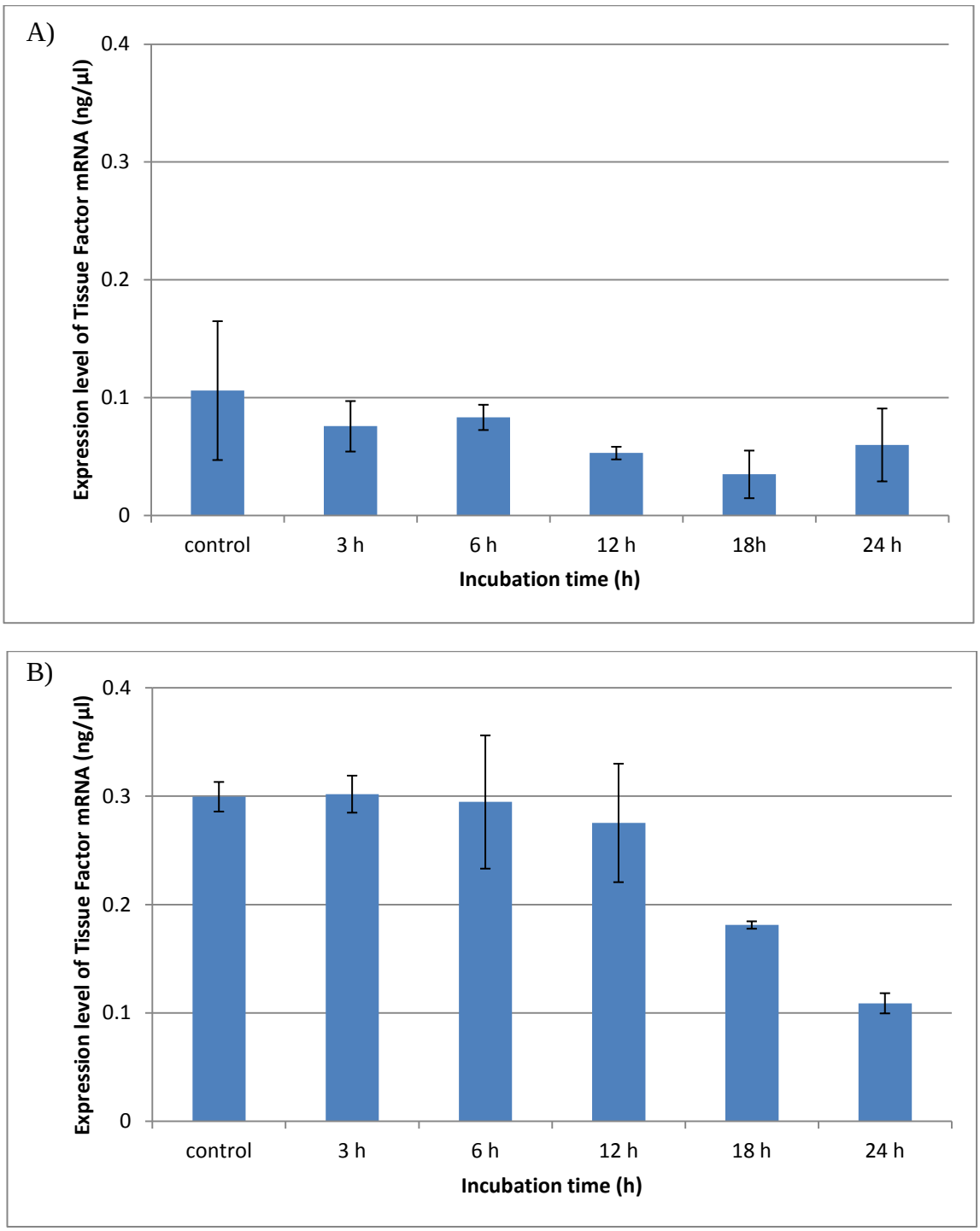

Figure 3.9: Analysis of the expression of tissue factor mRNA in HDBEC following the incubation with tumour-derived microparticles.

HDBEC were treated with tissue factor-bearing or tissue factor-deficient microparticles up to 24 h. Total RNA was then isolated from HDBEC and the amount of tissue factor mRNA determined by quantitative RT-PCR. The values were normalised against mRNA levels of housekeeping gene β-actin analysed by RT-PCR alongside. The normalised mRNA values (± 0.3) for (A) HDBEC treated with tissue factor-bearing microparticles (n=2) (mean SE±0.01) and (B) HDBEC treated with tissue factor-deficient microparticles were calculated (n=2) (mean SE±0.22).

3.7 Analysis of the internalisation of tissue factor by endothelial cells

In order to examine whether tissue factor-bearing microparticles are internalised by endocytosis, endothelial cells were pre-treated with the dynamin inhibitor, Dynasore (15 μM) prior to the incubation with tissue factor-bearing microparticles. Dynasore is a small GTPase inhibitor that blocks dynamin-dependent endocytosis by targeting dynamin-1 and dynamin2. This in turn result in the scission of endocytic vesicles. Cell surface tissue factor antigen was labelled with FITC-conjugated anti-tissue factor antibody and examined using confocal microscopy at intervals up to 2.5 h. Inclusion of Dynasore resulted in a slower rate of decline in cell surface tissue factor antigen, compared to the cells without Dynasore over 60 min incubation. Importantly, no significant tissue factor antigen was detected on endothelial cells following 120 min incubation with tissue factor bearing microparticles on pre-treatment with Dynasore, compared to the sample devoid at Dynasore treatment (Figure 3.10).

3.8 Cloning of recombinant tissue factor into pEGFP-C3 plasmid

An attempt was made to clone the tissue factor cDNA of 789 bp (see Figure 1.4) into the mammalian expression vector pEGFP-C3 (Figure 3.11), to overexpress tissue factor with enhanced green fluorescent protein (EGFP) on the N-terminal in MDA-MB-231 cell line. EGFP, which act as an expression marker, is composed of 238 amino acid residues that exhibit green fluorescence when exposed to light in the blue to ultraviolet range. EGFP-labelled tissue factor-bearing microparticles isolated from the transfected cells were intended to be used to monitor the trafficking of tissue factor within endothelial cells. Tissue factor DNA was amplified by PCR from a plasmid template (pCMV-XL5-TF) which already containing the tissue factor cDNA. In order to determine the optimal concentration of $MgCl_2$ for the PCR, the reaction was carried out at a range of concentrations of $MgCl_2$ (2-4 mM) and the PCR products were examined by agarose gel electrophoresis.

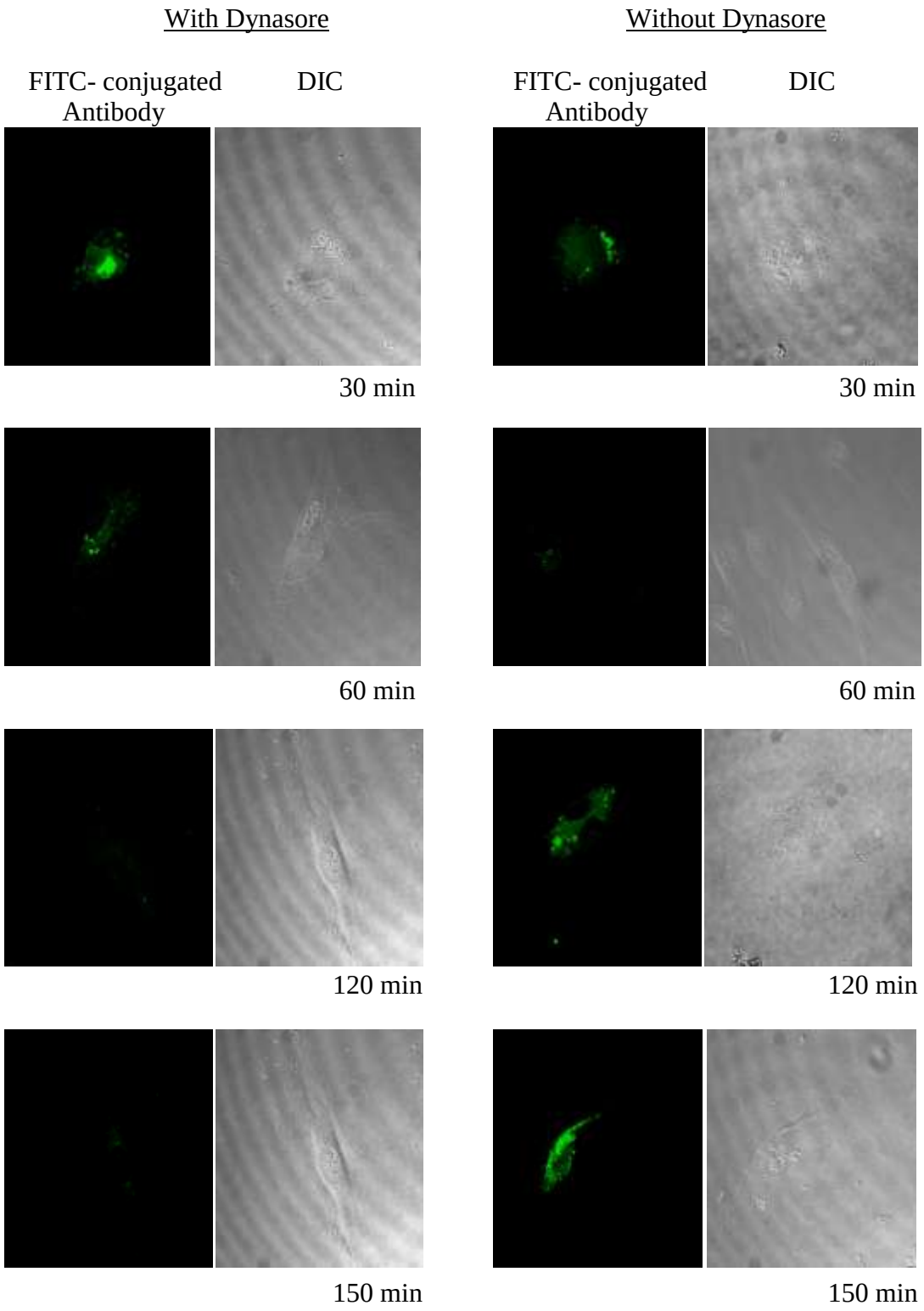

Figure 3.10: Analysis of the internalisation of tissue factor by endothelial cells.

HDBEC were treated with Dynasore prior to incubation with tissue factor-bearing microparticles for 2.5 h. A set of untreated (without Dynasore) HDBEC cells were included. HDBEC were then fixed with 3 % formaldehyde and washed with PBS. Cells surface tissue factor was labelled with an FITC-conjugated tissue factor antibody and analysed by confocal microscopy. Differential interference contrast microscopic images are shown alongside (magnification x63).

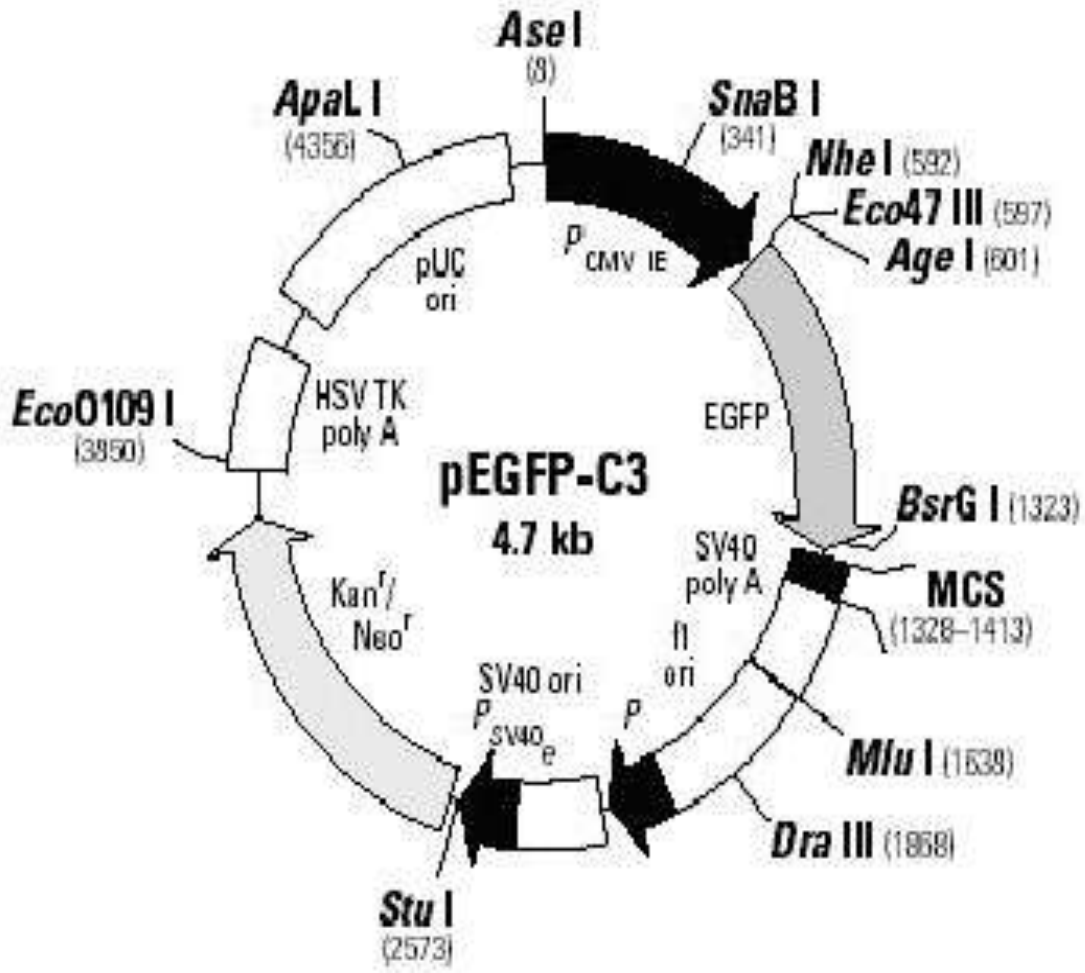

Figure 3.11: The structure of pEGFP-C3 mammalian expression vector

The pEGFP-C3 is a 4.7 kb long mamalian expression vector encoding a red-shifted variant of wild-type GFP which emits fluorescence (Excitation maximum = 488 nm, emission maximum = 507 nm). The multiple cloning sites in pEGFp-C3 is between the EGFP coding sequence and the SV40 poly A.

Examination of the PCR products showed a single band of approximately 800 bp, corresponding to the tissue factor cDNA in all samples (Figure 3.12). In the subsequent cloning procedure, 2 mM of $MgCl_2$ was used to avoid the possibility of non-specific DNA amplification.

pEGFP-C3 DNA plasmid was purified from bacterial culture using the Wizard Midiprep system and analysed by agarose gel electrophoresis. Examination of the plasmid DNA showed two bands of 4700 bp and 9000 bp, corresponding to the supercoiled and relaxed form of the plasmid respectively (Figure 3.13).

In order to insert tissue factor cDNA into the plasmid pEGFP-C3, tissue factor DNA and pEGFP-C3 were both digested using BamHI and HindIII restriction enzymes. The tissue factor DNA was then inserted into plasmid pEGFP-C3 by DNA ligation as described in section 2.10.7.

The concentration of Kanamycin for cell selection was optimised prior to bacteria transformation. TB1 *E.*coli cells were cultured overnight at 37 ºC, in Luria Broth medium with a range of concentrations of Kanamycin (0-80 µg/ml) and the cell density was then determined by measuring the absorption at 590 nm. No significant changes were observed in the TB1 cultures when treated with 30-40 µg/ml Kanamycin, compared to non-treated TB1 culture (Figure 3.14). In contrast, the growth of TB1 was greatly suppressed to less than 0.05 O.D on treatment with 50-80 µg/ml Kanamycin (Figure 3.13), indicating that 50 µg/ml is the lowest concentration of Kanamycin suitable for the selection of transformed bacteria. and colonies selected on Luria Broth

agar plate containing 50 µg/ml of Kanamycin. The recombinant plasmid DNA was then extracted and sequenced. The sequence of the recombinant plasmid DNA was:

BamHI
TGGGATCCTAATTTACTTGGAATCAACTAATTTCAAGACAATTTTGGAGTGG
GAACCCAAACCCGTCAATCAAGTCTACACTGTTCAAATAAGCACTAAGTCA
GGAGATTGGAAAAGCAAATGCTTTTACACAACAGACACAGAGTGTGACCTC
ACCGACGAGATTGTGAAGGATGTGAAGCAGACGTACTTGGCACGGGTCTTC
TCCTACCCGGCAGGGAATGTGGAGAGCACCGGTTCTGCTGGGGAGCCTCTG
HindIII
TATGAGAACTCCCCAAAGCTTTATTTTTTTTTTTTATTTTTTAGTTTAACCTCG
GACAGCCTACATTTCTTAATTT

The expected sequence of tissue factor DNA was shown in between the BamHI and HindIII restriction sites, indicating successful cloning of the tissue factor cDNA into the pEGFP-C3 mammalian expression vector. However, due to time constraints, this hybrid protein was not transfected and over-expressed in MDA-MB-231 cells for the isolation of GFP-labelled microparticles which could be used in the monitoring of tissue factor trafficking within endothelial cells.

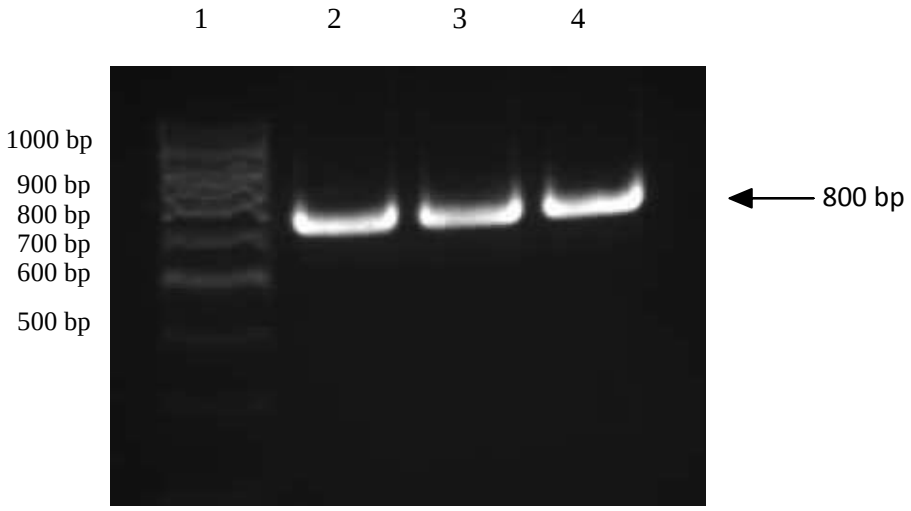

Figure 3.12: Determination of the optimal concentration of $MgCl_2$.

Tissue factor cDNA was amplified using pCMV-XL5-TF plasmid as template. PCR of tissue factor cDNA was carried out for 25 cycles at an annealing temperature of 62 °C at a range of $MgCl_2$ concentrations. The amplified DNA sequence was examined using 1.5 % (w/v) agarose gel electrophoresis and viewed using a UV transluminator. Lane 1: 100 bp DNA size marker, lane 2: 2 mM $MgCl_2$, lane 3: 3 mM $MgCl_2$, lane 4: 4 mM $MgCl_2$.

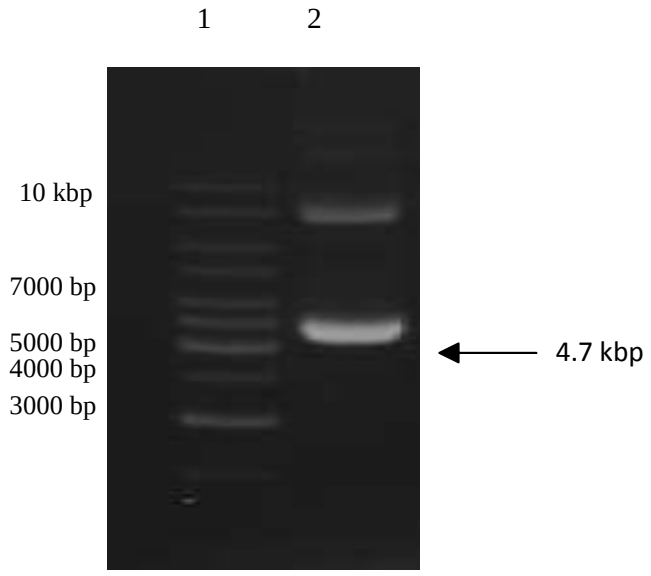

Figure 3.13: Examination of the mammalian expression vector, pEGFP-C3.

TB1 *E.*coli colonies containing pEGFP-C3 were cultured in 50 ml Luria Broth medium and DNA was extracted using the Wizard Midiprep system. The extracted plasmid DNA was examined using 0.5 % (w/v) agarose gel electrophoresis and viewed on a UV transluminator. Lane 1: 1 kbp DNA ladder, lane 2: pEGFP-C3 DNA plasmid.

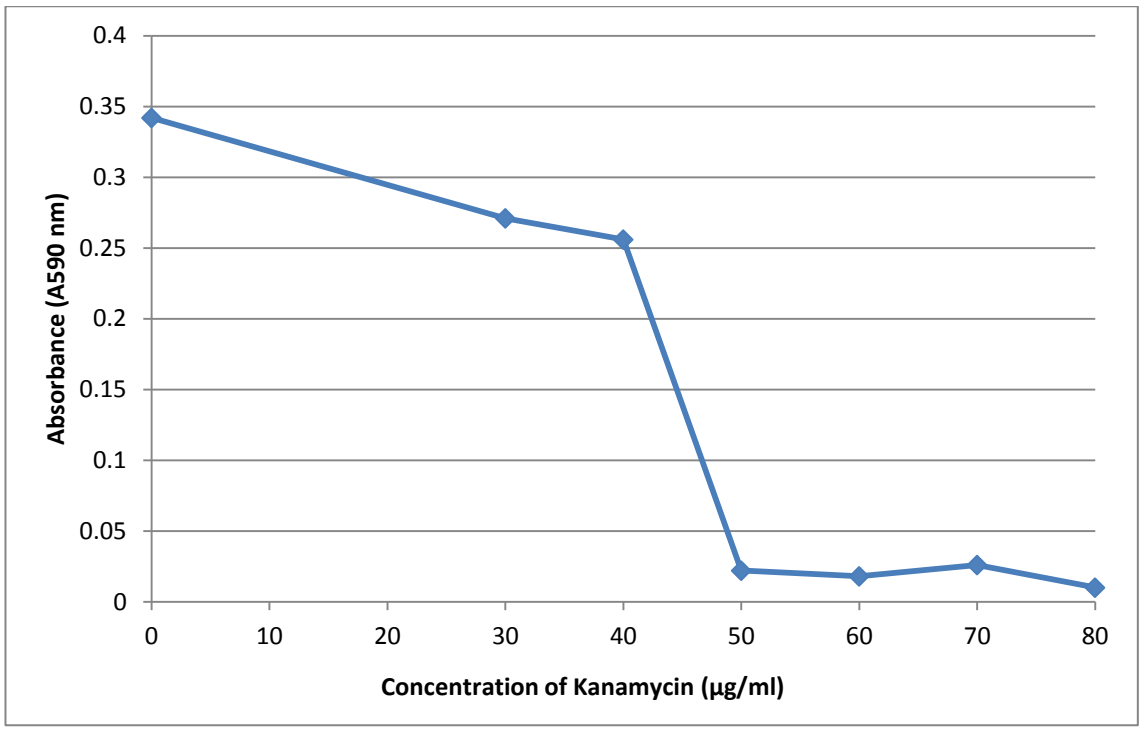

Figure 3.14: Determination of the optimal concentration of Kanamycin for cell selection.

TB1 *E.*Coli was cultured Luria Broth medium at a range of concentrations of Kanamycin (0-80 µg/ml). Cell density of TB1 was then quantified by measuring the absorbance at 590 nm using spectrophotometer (n=1).

Chapter 4

Discussion

Increased levels of tissue factor bearing-microparticles have been reported in the plasma of many cancer patients and particularly, in cancer patients who developed VTE. At high concentration, these tissue factor-bearing microparticles accumulate on the endothelial layer within blood vessel, and may cause vascular damage, induce angiogenesis and promote a hypercoagulable state (Tesselaar *et al.* 2007, Davila *et al.* 2008, Zwicker 2008, Aharon & Brenner 2009). Although tissue factor has been shown to be present within the endothelial layer in cancer patients, the source of this tissue factor still remains unknown. However, it has been reported that tissue factor can be transferred between various types of cells by microparticles (Scholz *et al.* 2002, Rauch *et al.* 2000). By xenografting human tumour cells into mice, Yu *et al.* (2008) showed the presence of human tissue factor within blood vessels suggesting that tissue factor can be transferred between tumour cells and the endothelium. In addition, cellular-derived microparticles have been shown to up-regulate tissue factor expression in endothelial cells (Hugel *et al.* 2005, Mesri & Altiery 1999, Aharon 2008). Therefore, it has been suggested that tumour cells may directly or indirectly be the main source of tissue factor-bearing microparticles which can give rise to prothrombic state in cancer patients (Del Conde *et al.* 2007, Davila *et al.* 2008). In this study, the source of the tissue factor, associated with endothelial cells was examined *in vitro*.

Microparticles derived from MDA-MB-231 and MCF-7 cells were taken up and accumulated by endothelial cells within 30 min of incubation (Figure 3.4). This is in line with results reported by Dasgupta *et al.* (2012) which show that the uptake of platelet-derived microparticles by human umbilical vein endothelial cells (HUVEC) occurred within 30 min of incubation. The interaction of tissue factor-bearing microparticles (obtained from MDA-MB-231 cells) with endothelial cells was also reflected in the presence of tissue factor antigen on the endothelial cell surface (Figure

3.5A). However, the surface tissue factor activity did not correlate with the concentration of tissue factor antigen; a disproportionately low level of tissue factor activity was detected with a high level of tissue factor antigen at 30 min (Figure 3.5A & 3.5B). A possible explanation for the lack of tissue factor activity at 30 min may involve the internalisation of phosphatidylserine from the outer surface of the endothelial membrane, following the fusion of microparticles with the endothelial cell membrane. Phosphatidylserine has previously been shown to aid the fusion of microparticles with cell membranes and is rapidly internalised (Köppler *et al.* 2006). This is supported by the disappearance of annexin v-labelling from the surface of endothelial cells (Figure 3.8). Therefore, since the anionic phospholipids are required for the initiation and propagation of tissue factor-dependent coagulation (Tesselaar *et al.* 2007), internalisation of phosphatidylserine explains the reduced thrombin generation capacity observed.

Interestingly, the level of detectable tissue factor antigen rapidly decreased after 30 min incubation, indicating the possible internalisation of tissue factor. Moreover, tissue factor antigen reappeared on the surface of endothelial cells after 120 min incubation and persisted for a further 240 min. The initial binding of tissue factor to the cell surface at 30 min incubation and the reappearance of tissue factor on the surface of endothelial cell at 120 min post-incubation was further confirmed using an anti-tissue factor FITC-conjugated antibody (Figure 3.7). This data suggests that tissue factor may be recycled back to the endothelial cell surface. Importantly, the reappearance of the tissue factor antigen was accompanied with substantial tissue factor activity, although this activity subsided after 150 min incubation (Figure 3.5B). Furthermore, this activity was accompanied with the substantial exposure of phosphatidylserine at 90 min and significantly increased at 120 min (Figure 3.8). This exposure of phosphotidylserine

may occur as a consequence of the activation of endothelial cells by cancer cell-derived microparticles, which leads to the membrane remodelling within endothelial cells. This membrane modification in turn results in the exposure of anionic phospholipids, and specifically phosphatidylserine on the surface of the endothelial cells leading to the enhancement of the thrombin-generation capacity of on the surface of endothelial cells (Nomura *et al.* 2008). However, while a significant degree of cell-surface phosphatidylserine was detectable as early as 90 min post-incubation, this was not accompanied with significant tissue factor activity and therefore suggests that both tissue factor and phosphatidylserine are essential for the increased proccoagulant activity at the cell surface (Figure 3.5A & 3.8). It is known that tissue factor and phosphatidylserine act synergistically as potent triggers of the blood coagulation cascade (Morrissey 2001). It is also noteworthy that the tissue factor has to reach a concentration threshold in order to initiate the coagulation activity (Jesty *et al.* 2005). In contrast, incubation of endothelial cells with MCF-7 cell-derived tissue factor-deficient microparticles did not produce any detectable cell surface tissue factor antigen or activity throughout the incubation period (Figure 3.6A & 3.6B).

Endothelial cells do not normally express detectable amounts of tissue factor but have been shown to capable of expressing this protein in response to inflammatory mediators such as tumour necrosis factor (TNF), lipopolysaccharide (LPS) (Parry & Mackman 1995). In order to determine if the reappearance of tissue factor on the cell surface was a consequence of *de novo* expression of tissue factor in response to tumour-derived microparticles, the level of mRNA was quantified using a tissue-factor specific quantitative RT-PCR. Examination of untreated HDBEC confirmed the lack of tissue factor mRNA in these cells. Furthermore, incubation of HDBEC with microparticles from either MDA-MB-231 or MCF-7 cell lines did not result in the up-regulation of

tissue factor mRNA when monitored up to 24 h (Figure 3.9). These data suggests that the increase in endothelial cell surface tissue factor following incubation with tissue factor-containing microparticles is due to the transfer of tissue factor rather than *de novo* tissue factor expression. This is also in agreement with the report by Yu *et al.* (2008) who showed that incubation of mouse endothelial cells with tissue factor-bearing microparticles obtained from cancer cells, resulted in increased tissue factor antigen and procoagulant activity on mouse endothelial cells. Therefore, tumour-derived tissue factor-bearing microparticles appear to be the main source of tissue factor in the endothelial cells and may contribute to cancer-associated thrombosis.

The disappearance of tissue factor at 30 min and subsequent reappearance of tissue factor at 120 min on the surface of endothelial cells suggests that tissue factor was internalised by endothelial cells and recycled back to the cell surface (Figure 3.5A). The internalisation of tumour derived-microparticles by endothelial cells was previously shown to occur through an endocytotic process (Kawamoto *et al.* 2012). Furthermore, endothelial cells have been shown to take up platelet microparticles specifically through clathrin-dependent endocytosis (Dasgupta *et al.* 2012). To investigate the internalisation of tissue factor, endothelial cells were pre-treated with a dynamin inhibitor dynasore, followed by the incubation with tissue factor-bearing microparticles. In these cells, the uptake of tissue factor was slowed down compared to untreated cells and was indicated by the slower rate of disappearance of tissue factor from the endothelial cell surface at 60 min post-incubation with microparticles (Figure 3.10). However, since the fluorescence signal eventually diminished, it may be assumed that tissue factor was either internalised, degraded or lost from the endothelial cell surface. More importantly, tissue factor antigen did not reappear at 120 min post-incubation as observed in the untreated cells (Figure 3.10). The recycling of endogeneous tissue factor, measured as

FVIIa activity has previously been demonstrated in BHK cells (Hansen *et al*. 2001). Moreover, it has been shown that the re-appearance of the endogenous tissue factor on the cell membrane may be accompanied with substantial TF-FVIIa activity (Hansel *et al.* 2001). Therefore, endothelial cells may also have the ability to take up microparticle-associated tissue factor and recycle this internalised tissue factor in active form on the endothelial cell surface.

The potential role of tumour-derived microparticles in the development of cancer-related thrombosis was shown *in vitro*. Elevated levels of circulating tissue factor-bearing microparticles have been reported in cancer patients and associated with increased risk of thrombosis (Khorona *et al.* 2008, Zwicker 2008, Auwerda *et al.* 2011). A major proportion of the circulating tissue factor-bearing microparticles detected in cancer patients have been suggested to arise from tumour cells (Zwicker 2008). These microparticles have been suggested to act as a carrier of tissue factor and can mediate the transfer of tissue factor between tumour cells and endothelial cells, increasing the concentration of tissue factor within endothelial cells (Yu *et al.* 2008, Davila *et al.* 2008). In addition, others have suggested that endothelial cells are able to express tissue factor protein in response to cytokines (Zwicker *et al.* 2011), and the release of cytokines by endothelial cells can be triggered by microparticles (Nomura *et al.* 2001). Therefore, the source of tissue factor which contribute to the development of VTE remains unidentified. In this study, the presence of tissue factor within endothelial cells was shown as a consequence of the transfer of tissue factor from tumour-derived tissue factor-bearing microparticles rather than the *de novo* expression. Accumulation and localisation of these tissue factor-bearing microparticles on endothelial cells can alter the haemostatic balance and consequently increase the thrombogenicity of endothelial cells (Davila *et al.* 2008, Zwicker *et al.* 2009). Furthermore, the ability of tumour-

derived microparticles to transfer tissue factor between cells may explain why cancer patients often experience thrombosis at a distal site of the underlying tumour. For example, the majority of the pancreatic cancer patients are reported to develop pulmonary embolism (Khorona & Fine 2004). This suggests that microparticles can act as vehicles to transfer tissue factor from tumour cells to endothelial cells, leading to thrombosis. Importantly, this study has demonstrated that endothelial cells are capable of accumulating tissue factor from microparticles which in turn leads to the recycling of this internalised tissue factor on the surface of endothelial cells in a highly active form. Accumulation of this active tissue factor on the surface of endothelial cells, together with the exposure of phosphatidylserine can result in the rapid increase in the local procoagulant activity. This may be the underlying cause of activation of coagulation on the intact endothelium and contribute to the development of VTE in cancer patients.

The recycling of tissue factor by endothelial cells resulted in a delay in the reappearance of tissue factor activity on the surface of endothelial cells which may explain some of the discrepancies between the level of tissue-factor bearing microparticles measured in patients and the incidence of thrombosis observed. A previous report by Zwicker *et al.* (2009) showed that 34.8 % of cancer patients with measurable tissue factor-bearing microparticles developed a thrombotic event within one year following enrolment into the study. In contrast, no incidence of VTE was reported in cancer patients without measurable tissue factor-bearing microparticles (Zwicker *et al.* 2009). In fact, the presence of tissue factor-bearing microparticles in cancer patients predicted a seven-fold increased risk of VTE, compared to cancer patients who were negative to tissue factor-bearing microparticles (Zwicker *et al.* 2009). In addition, Khorona *et al.* (2008) measured the microparticle-derived tissue factor antigen in cancer patients, at weekly intervals, and reported that tissue factor antigen remains low in patients who do not

develop VTE over the observation period. Interestingly, in cancer patients who developed VTE, a sudden rise in circulating microparticle-derived tissue factor antigen was observed prior to the development of VTE. However, there were wide variations in the concentrations of microparticle-derived tissue factor antigen detected in these cancer patients, ranging from 250 to 500 pg/ml (Khorona *et al.* 2008). Importantly, only 27 % of the cancer patients with high levels of circulating tissue factor developed VTE (Khorona *et al.* 2007, Uno *et al.* 2007). Therefore, while these observations suggest that the presence of tissue-factor bearing microparticles may be a potential biomarker for the incidence of VTE, the risk of VTE does not directly correlate with the concentration of circulating microparticles.

The uptake of microparticles by endothelial cells through endocytosis has been demonstrated previously (Kawamoto *et al.* 2012). This work has shown that the tissue factor component may be recycled to the cell surface. To further establish this process, the trafficking of tissue factor within the endothelial cells needs to be monitored. In order to do so, HDBEC may be treated with GFP-labelled tissue factor-bearing microparticles. GFP-labelled microparticles can be isolated from MDA-MB-231 cells, which have been transfected with and over expressed the hybrid protein with GFP on the N- (EGFP-TF) or the C-terminal (TF-tGFP) of tissue factor. Subsequently, at intervals, antibody against the Rab proteins may be applied to detect endogeneous levels of the Rab family proteins. The Rab family of proteins are involved in a number of vesicle-mediate transports. For example, Rab 4 is associated with early endosomes and the recycling endosomes which function as a key regulator for the recycling of proteins, while Rab 5 functions as a key regulator of vesicle trafficking during early endocytosis. Consequently, analysis on the co-localization of GFP-labelled tissue factor and the Rab proteins may help to determine the fate of the internalised tissue factor. In conclusion,

this study has shown that tissue factor can be internalised by endothelial cells, on exposure to tumour-derived microparticles. The tissue factor component may then be recycled back to the cell surface in a highly active form.

Chapter 5

References

Abe K, Shoji M, Chen J, Bierhaus A, Danave I, Micko C, Casper K, Dillehay DL, Nawroth PP, Rickles FR. Regulation of vascular endothelial growth factor production and angiogenesis by the cytoplasmic tail of tissue factor. *Proc Natl Acas Sci USA.* 1999; **96**: 8663-8668.

Aharon A, Brenner B. Microparticles, thrombosis and cancer. *Best Pract Res Clin Haematol.* 2009; **22**: 61-69.

Auwerda JJ, Yuana Y, Osanto S, de Maat MP, Sonneveld P, Bertina RM, Leebeek FW. Microparticle-associated tissue factor activity and veneous thrombosis in multiple myeloma. *Thromb Haemost.* 2011; **105**: 14-20.

Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci USA.* 1990; **87**: 6934-6938.

Bevers EM, Comfurius P, Dekkers DW, Zwaal RF. Lipid translocation across the plasma membrane of mammalian cells. *Biochim Biophys Acta.* 1999; **1439**: 317-330.

Bromberg ME, Sundaram R, Homer RJ, Garen A, Konigsberg WH. Role of tissue factor in metastasis: functions of the cytoplasmid and extracellular domains of the molecule. *Thromb Haemost.* 1999; **82**: 88-92.

Burnier L, Fontana P, Kwak BR, Angelillo-Scherrer A. Cell-derived microparticles in haemostasis and vascular medicine. *Thromb Haemost.* 2009; **101**: 439-451.

Camerer E, Gjernes E, Wiiger M, Pringle S, Prydz H. Binding of factor VIIa to tissue factor on keratinocytes induces gene expression. *J Biol Chem.* 2000; **275**: 6580-6585.

Camerer E, Rottingen JA, Gjernes E, Larsen K, Skartlien AH, Iversen JG, Prydz H. Coagulation factors VIIa and Xa induce cell signaling leading to up-regulation of the egr-1 gene. *J Biol Chem.* 1999; **274**: 32225-32233.

Chandradas S, Deikus G, Tardos JG, & Bogdanov VY. Antagonistic roles of four SR proteins in the biosynthesis of alternatively spliced tissue factor transcripts in monocytic cells. *J Leukoc Biol.* 2010; **87**:147–152.

Chu AJ. Tissue factor, blood coagulation, and beyond: an overview. *Int J Inflam.* 2011; **2011**: 367284.

Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 2009; **19**: 43-51.

Conde-Vancells J, Rodriguez-Suarez E, Embade N, Gil D, Matthiesen R, Valle M, Elortza F, Lu SC, Mato JM, Falcon-Perez JM. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. *J Proteome Res.* 2008; **7**: 5157-5166.

Contrino J, Hair G, Kreutzer DL, Rickles FR. In situ detection of tissue factor in vascular endothelial cells: correlation with the malignant phenotype of human breast disease. *Nat Med.* 1996; **2**: 209-215.

Dasgupta SK, Le A, Chavakis T, Rumbaut RE, Thiagarajan P. Developmental endothelial locus-1 (Del-1) mediates clearance of platelet microparticles by the endothelium. *Circulation.* 2012; **125**: 1664-1672.

Davila M, Amirkhosravi A, Coll E, Desai H, Robles L, Colon J, Baker CH, Francis JL. Tissue factor-bearing microparticles derived from tumor cells: impact on coagulation activation. *J Thromb Haemost.* 2008; **6**: 1517-1524.

Del conde I, Shrimpton CN, Thiagarajan P, Lopez JA. Tissue factor-bearing microvesicles arise from lipid rafts and fuse with activated platelet to initiate coagulation. *Blood.* 2005; **106**: 1604-1611.

Dignat-George F, Boulanger CM. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Boil.* 2011; **31**: 27-33.

Dvorak HF, Van DeWater L, Bitzer AM, Dvorak AM, Anderson D, Harvey VS, Bach R, Davis GL, DeWolf W, Carvalho AC. Procoagulant activity associated with plasma membrane vesicles shed by cultured tumor cells. *Cancer Res.* 1983; **43**: 4434-4442.

Eisenreich A, Rauch U. Regulation and differential role of the tissue factor isoforms in cardiovascularbiology. *Trens Cardiovas Medi.* 2010; **20**: 199-203.

Falangar A. Thrombophilia in cancer. *Semin Thromb Hemost.* 2005; **31**: 104-110.

Falati S, Liu Q, Gross P, Merrill-Skoloff G, Chou J, Vandendries E, Celi A, Croce K, Furies BC, Furie B. Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. *J Exp Med.* 2003; **197**: 1585-1598.

Fei H, Berliner JA, Purhami F, Drake TA. Regulation of endothelial cell tissue factor expression by minimally oxidized low density lipoprotein and lipopolysaccharide. *Arterioscler Thromb.* 1993; **13**: 1711-1717

Fevrier B, Raposo G. Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr Opin Cell Biol.* 2004; **16**: 415-421.

Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, Weber M, Hamm CW, Roxe T, Muller-Ardogan M, Bonauer A, Zeihei AM, Dimmeler S. Circulating microRNAs in patients with coronary artery disease. *Circ Res.* 2010; **107**: 677-684.

Forster Y, Meye A, Albrecht, Schwenzer B. Tissue factor and tumor: clinical and laboratory aspects. *Clin Chim Acta.* 2006; **364**: 12-21.

Fukushima Y, Nakanishi M, Nonogi H, Goto Y, Iwai N. Assessment of plasma miRNAs in congestive heart failure. *Circ J.* 2011; **75**: 336-340.

Furie B, Furie BC. Cancer-associated thrombosis. *Blood Cells Mol Dis.* 2006; **36**: 177-181.

Gilbert GE, Sim PJ, Wiedmer T, Furie B, Furie BC, Shattil SJ. Platelet-derived microparticles express high affinity receptors for factor VIII. *J Biol Chem.* 1991; **266**: 17261-17268.

Hansen CB, Pyke C, Petersen LC, Rao LV. Tissue factor-mediated endocytosis, recycling, and degradation of factor VIIa by a clathrin-independent mechanism not requiring the cytoplasmic domain of tissue factor. *Blood.* 2001; **97**: 1712-1720.

Haver VG, Slart RH, Zeebregts CJ, Peppelenbosch MP, Tio RA. Rupture of vulnerable atherosclerotic plaques: microRNAs conducting the orchestra*? Trends Cardiovas Med.* 2010; **20**: 65-71

Heit JA. Cancer and venous thromboembolism: scope of the problem. *Cancer Control.* 2005; **12**: 5-10.

Hron G, Kollars M, Weber H. Tissue factor- positive microparticles: cellular origin and assodication with coagulation activation in patients with colorectal cancer. *Thromb Haemost.* 2007; **97**: 119-123.

Hugel B, Martinez MC, Kunzelmann C, Freyssinet JM. Membrane microparticles: two sides of the coin. *Physiology (Bethesda).* 2005; **20**: 22-27.

Jesty J, Beltrami E. Positive feedbacks of coagulation: their role in threshold regulation. *Arterioscler Thromb Vasc Biol.* 2005; **25**: 2463–2469.

Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, Ahn YS. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res.* 2003; **109**: 175-180.

Kakkar AK, Lemoine NR, Scully MF, Tebbutt S, Williamson RC. Tissue factor expression correlates with histological grade in human pancreatic cancer. *Br J Surg*. 1995; **82**: 1101-1104.

Kao FT, Hartz J, Horton R, Nemerson Y, Carson SD. Regional assignment of human tissue factor gene (F3) to chromosome 1p21-22. *Somat Cell Mol Genet.* 1988; **14**: 407-410.

Kawamoto T, Ohga N, Akiyama K, Hirata N, Kitahara S, Maishi N, Osawa T, Yamamoto K, Kondoh M, Shindoh M, Hida Y, Hida K. Tumor-derived microvesicles induce proangiogenic phenotype in endothelial cells via endocytosis. *PLoS One.* 2012; **7**: e34045.

Kawashima T, Shioi T. MicroRNA, emerging role as a biomarker of heart failure. *Circ J.* 2011; **75**: 268-269.

Khorona AA, Ahrendt SA, Ryan CK, Francis Cw, Hruban RH, Hu YC, Hostetter G, Harvey J, Taubman MB. Tissue factor expression, angiogenesis and thrombosis in pancreatic cancer. *Clin Cancer Res.* 2007; **13**: 2870-2875.

Khorona AA, Fine RL. Pancreatic cancer and thromboembolic disease. *Lancet Oncol.* 2004; **5**: 655-663.

Khorona AA, Francis CW, Manzies KE, Wang JG, Hyrien O, Hathcock J, Mackman N, Taubman MB. Plasma tissue factor may be predictive of veneous thromboembolism in pancreatic cancer. *J Thromb Haemost.* 2008; **6**: 1983-1985.

Lechner D, Weltermann A. Chemotherapy-induced thrombosis: a role for microparticles and tissue factor? *Semin Thromb Hemost.* 2008; **34**: 199-203.

Leroyer AS, Anfosso F, Lacroix R, Sabatier F, Simoncini S, Njock SM, Jourde N, Brunet P, Camoin-Jau L, Sampol J, Dignat-George F. Endothelial-derived microparticles: biological conveyors at the crossroad of inflammation thrombosis and angiogenesis. *Thromb Haemost.* 2010; **104**: 456-463.

Mackman N, Tilley RE, Key NS. Role of extrinsic pathway of blood coagulation in hemostasis and thrombosis. *Arterioscler Thromb Vasc Biol.* 2007; **27**: 1687-1693.

Mackman N. Regulation of tissue factor gene. *Thromb Heamost.* 1997; **78**:747-754

Mackman N. Role of tissue fator in hemostasis, thrombosis, and vascular development. *Arterioscler Thromb Vasc Biol.* 2004; **24**: 1015-1022.

Martinez MC, Tesse A, Zobairi F, Andraianstsitohaina R. Shed membrane microparticles from circulating and vascular cells in regulating vascular function. *Am J Physiol Heart Circ Physiol.* 2005; **288**: 1004-1009.

Mechtcheriakova D, Schabbauer G, Lucerna M, Clauss M, De Martin R, Binder BR, Hofer E. Specificity, diversity, and convergence in VEGF and TNF-alpha signalling events leading to tissue factor up-regulation via EGR-1 in endothelial cells. *Faseb J.* 2001. **15**: 230-242

Mesri M, Altieri DC. Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signalling pathway. *J Biol Chem.* 1999; **274**: 23111-23118.

Milsom C, Yu J, May L, Mrrhan B, Magnus N, Al-Nedawi K, Luyendyk J, Weotz J, Klement P, Broze G, Mackman N, Rak J. The role of tumour- and host-related tissue factor pools in oncogene- driven tumour progression. *Thromb Res.* 2007; **2**: s82-s91.

Morel O, Jesel L, Freyssinet JM, Toti F. Cellular mechanism underlying the formation of circulating microparticles. *Arterioscler Thromb Vasc Biol.* 2011; **31**: 15-26.

Morrissey JH. Tissue factor: an enzyme cofoactor and a true receptor. *Thromb Haemost.* 2001; **86**: 66-74.

Nemerson Y. Tissue factor: then and now. *Thromb Haemost.* 1995; **74**: 180-184.

Nomura S, Ozaki Y, Ikeda Y. Function and role of microparticles in various clinical settings. *Thromb Res.* 2008; **10**: 1016-1031.

Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, Kambayashi J. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. *Atherosclerosis.* 2001; **158**: 277-287.

Ollivier V, Bentolila S, Chabbat J, Hakim J, de Prost D. Tissue factor-dependent vascular endothelial growth factor production by human fibroblasts in response to activated factor VII. *Blood.* 1998*;* **91***:* 2698-2703.

Parry GC, Mackman N. Transcriptional regulation of tissue factor expression in human endothelial cells. *Arterioscler Thromb Vasc Biol.* 1995; **15**: 612-621.

Parry GC, Mackman N. Transcriptional regulation of tissue factor expression in human endothelial cells. *Arterioscler Thromb Vasc Biol.* 1995; **15 (5)**: 612-21.

Pendurthi UR, Allen KE, Ezban M, Rao LVM. Factor VIIa and thrombin induce the expression of Cyr61 and connective tissue growth factor, extracellular matrix signaling proteins that could act as possible downstream mediators in factor VII.tissue factor-induced signal transduction. *J Biol Chem.* 2000*;* **275***:* 14632-14641.

Peppelenbosch MP, Versteeg HH. Cell biology of tissue factor, an unusual member of the cytokine receptor family. *Trends Cardiovasc Med*. 2001; **11**: 335-339.

Petersen LC, Valentin S, Hedner U. Regulation of the extrinsic pathway system in health and disease: the role of factor VIIa and tissue factor pathway inhibitor. Thromb Res. 1995; 1: 1-47

Rak J, Milsom C, Magnus N, Yu J. Tissue factor in tumour progression. *Best Pract Res Clin Haematol.* 2009; **22**: 71-83.

Rauch U, Bonderman D, Bohrmann B, Badimon JJ, Himber J, Riederer MA, Nemerson Y. Transfer of tissue factor from leukocytes to platelets is mediated by CD15 and tissue factor. *Blood*. 2000; **96**: 170-175.

Rickles FR, Patierno S, Fernandez PM. Tissue factor, thrombin and cancer. *Chest.* 2003; **124**: 58s-68s.

Rickles FR. Mechanisms of cancer-induced thrombosis in cancer. *Pathophysiol Haemost Thromb*. 2006: **35**: 103-110.

Sawada M, Miyake S, Ahdama S. Matsunara O, Masuda S, Yakumaru K, Yoshizawa Y. Expression of tissue factor in non-small-cell lung cancers and its relationship to metastasis. *Br J Cancer Res.*1999; **79**: 472-477.

Scarpati EM, Sadler JE. Regulation of endothelial cell coagulant properties. *J Biol Chem*. 1989; **264**: 20705-20713

Scholz T, Temmler U, Krause S, Heptinstall S, Lösche W. Transfer of tissue factor from platelets to monocytes: role of platelet-derived microvesicles and CD62P. *Thromb Haemost.* 2002; **88**: 1033-1038.

Sevitt S. The structure and growth of valve-pocket thrombi in femoral veins. *J Clin Pathol.* 1974; **27**: 517-528.

Sørensen HT, Mellemkjaer L, Olsen JH, Baron JA. Prognosis of cancers associated with venous thromboembolism. *N Engl J Med.* 2000; **343**: 1846-1850.

Srinivasan R, Bogdonov VY. Splice variants of tissue factor and integrin-mediated signalling. *Thromb Res.* 2012; **129**: s34-s37.

Tesselaar ME, Romijin FP, Van Der Linden IK, Prins FA, Bertina RM, Osanto S. Microparticles-associated tissue factor activity: a link between cancer and thrombosis? *J Thromb Haemost.* 2007; **5**: 520-527.

Thomas GM, Panicot-Dubois L, Lacroix R, Dignet-George F, Lombardo D, Bubois C. Cancer cell–derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. *J Exp Med.* 2009; **206**: 1913-1927.

Tilley RE, Holscher T, Belani R, Nieva J, Mackman N. Tissue factor activity is increased in a combined platelet and microparticle sample from cancer patients. *Thromb Res.* 2008; **122**: 604-609.

Trousseau A. Phlegmasia alba dolens. *Lectures on clinical medicine, delivered at the Hotel-Dieu, Paris.* 1865; **5**: 281-332

Tushuizen ME, Diamat M, Sturk A, Nieuwland R. Cell-derived microparticles in the pathogenesis of cardiovascular disease. Friend or Foe? *Arterioscler Thromb Vasc Biol.* 2011; **31**: 4-9.

Ueno T, Toi M, Koike M, Nakamura S, Tominaga T. Tissue factor expression in breast cancer tissues: its correlation with prognosis and plasma concentration. *Br J Cancer.* 2000; **83**: 164-170.

Uno K, Homma S, Satoh T, Nakanishi K, Abe D, Matsumoto K, Oki A, Tsunoda H, Yamaguchi I, Nagasawa T, Yoshikawa H, Aonuma K. Tissue factor expression as a possible determinant of thromboemlism in ovarian cancer. *Br J Cancer*. 2007; **96**: 290-295.

Vandendries ER, Furie BC, Furie B. Role of P-selectin and PSGL-1 in coagulation and thrombosis. *Thromb Haemost.* 2004; **92**: 459-466.

VanWijk MJ, VanBabel E, Sturk A, Niwuwland R. Microparticles in cardiovascular disease. *Cardiovasc Res.* 2003; **59**: 277-287.

Viles-Gonzalez JF, Badimon JJ. Artherothrombosis: the role of tissue factor. *Int J Biochem Cell Biol.* 2004; **36**: 25-30.

White RH, Chew H, Wun T. Targeting patients for anticoagulant prophylaxis trials in patients with cancer: who is at highest risk? *Thromb Res.* 2007; **120**: S29-40.

Yu G, Li H, Wang X, Wu T, Zhu J, Huang S, Wan Y, Tang J. MicroRNA-19a targets tissue factor to inhibit colon cancer cells migration and invasion. *Mol Cell Biochem*. 2013; **380** (1-2): 239–247, 2013.

Yu J, May L, Milsom C, Anderson GM, Weitz JI, Luyendyk JP, Broze G, Mackman N, Rak J. Contribution of host-derived tissue factor to tumor neovascularization. *Arterioscler Thromb Vasc Biol*. 2008; **28**: 1975-1981.

Yu X, Riley T, Levine AJ. The regulation of the endosomal compartment by p53 the tumour suppressor gene. *Febs J.* 2009; **276** (8): 2201-12.

Zhang X, Yu H, Lou JR, Zheng J, Zhu H, Popescu NI, Lupu F, lind SE, Ding WQ. MicroRNA-19 (miR-19) regulates tissue factor expression in breast cancer cells. *J Biol Chem.* **2011**; 286: 1429-1435.

Zwaal RA, Schroit AJ. Pathophysiology implications of membrane phospholipids asymmetry in blood cells. *Blood.* 1997; **89**: 1121-1132.

Zwicker JI, Liebman HA, Neuberg D, Lacroix R, Bauer KA, Furie BC, Furie B. Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. *Clin Cancer Res*. 2009; **15**: 6830-6840.

Zwicker JI, Trenor CC, Furie BC, Furie B. Tissue factor-bearing microparticles and thrombus formation. *Arterioscler Thromb Vasc Biol.* 2011; **31**: 728-733.

Zwicker JI. Tissue factor-bearing microparticles and cancer. *Semin Thromb Hemost.* 2008; **34**: 195-198.