THE UNIVERSITY OF HULL

Low molecular weight heparin downregulates tissue factor expression and activity by modulating growth factor receptor-mediated induction of nuclear factor κB

being a Thesis submitted for the Degree of

Masters by research

in the University of Hull

by

Donna Louise Fountain B.Sc (Hons)

Department of Biological Sciences

February 2013

ABSTRACT

The association between thrombosis and cancer has long been established. Tissue factor (TF), the initiator of the extrinsic pathway in the blood coagulation cascade, has been implicated as one of the most important physiological factors causing such complications. The expression of TF has been observed in many different types of cancer and its increased expression has been shown to correlate with increased incidence of thrombotic events in cancer patients. The use of traditional anticoagulants, such as warfarin, alongside cancer treatments has proved problematic, causing excess bleeding, and has not significantly reduced the risk of thrombosis. Over recent years treatment of cancer patients with low molecular weight heparin (LMWH) has been reported to have beneficial effects that not only reduce the risk of thrombosis but also increase the patient's life expectancy. This study aimed to examine the influence of a range of LMWH concentrations (0-2000μg/ml) on TF expression, activity, and cell invasiveness in five different cancer cells lines known to express high levels of TF. A decrease in TF mRNA, cellular TF antigen and TF activity was found to correlate with increasing concentrations of LMWH. Additionally LMWH was observed to downregulate nuclear factor κB (NFκB) activity in all cell lines. Further to this, TF expression was suppressed in the presence of LMWH when cells were supplemented with EGF, bFGF and VEGF. Finally, a decrease in cellular invasion was observed following treatment with increasing concentrations of LMWH. These results indicate that LMWH is capable of suppressing TF expression by downregulating NFκB activity through interference with mechanisms involving the action of growth factors. Furthermore, the results indicate that LMWH reduces the rate of cancer cell invasion through a mechanism which appears to be dependent on expression of TF.

PUBLICATIONS

Papers

Ettelaie, C., Fountain, D., Collier, M.E., Beeby, E., Xiao, Y.P & Maraveyas, A. (2011). Low molecular weight heparin suppresses tissue factor-mediated cancer cell invasion and migration *in vitro*. *Experimental and Therapeutic medicine,* 2, 363-367.

Ettelaie, C., Fountain, D., Collier, M.E., Elkeeb, A.M., Xiao, Y.P & Maraveyas, A. (2011). Low molecular weight heparin downregulates tissue factor expression and activity by modulating growth factor receptor-mediated induction of nuclear factor-kappaB. *Biochim Biophys Acta*, 1812, 1591-600.

The contributions of each author;

Ettelaie, C - designed the experiments, carried out some of the experiments, interpreted the data and wrote the papers.

Fountain, D - designed the experiments, carried out the experiments, interpreted the data and was involved in the writing of the papers.

Collier, M.E - designed the experiments, interpreted the data and was involved in the writing of the papers.

Beeby, E – carried out some of the experiments.

Elkeeb, A.M - was involved in the writing of the papers.

Xiao, Y.P - was involved in the writing of the papers.

Maraveyas, A - designed the experiments and was involved in the writing of the papers.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr Camille Ettelaie for her guidance and support throughout this project, in particular for her help with the statistical analysis of the results. I would also like to thank Mary Collier for her help during this project. I would like to thank my parents for their continuous encouragement and for their support in making it possible to continue my studies. Finally I would like to thank all my friends for their encouragement throughout this project.

To my parents

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LIST OF ABBREVIATIONS

AP-1	Activator Protein - 1
bp	Base pairs
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
°C	Degrees centigrade
DMSO	Dimethyl sulphoxide
$CaCl_2$	Calcium chloride
cDNA	Copy deoxyribonucleic acid
CH_3COONa	Sodium acetate
CO_2	Carbon dioxide
Da	Daltons
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonuclease
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid disodium salt
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
EMEM	Eagles minimum essential medium
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FIX	Factor IX
FIXa	Activated factor IX
FV	Factor V

FVa	Activated factor V
FVII	Factor VII
FVIIa	Activated factor VII
FVIII	Factor VIII
FVIIIa	Activated factor VIII
FX	Factor X
FXa	Activated factor FX
FXI	Factor XI
FXIa	Activated factor XI
FXII	Factor XII
FXIIa	Activated factor XII
g	Gram
g	Gravity
G	Guanine
h	Hour
HRP	Horseradish peroxidise
IgG	Immunoglobulin G
KCl	Potassium chloride
L	Litre
LB	Luria-Bertani
Log	Logarithm
M	Molar
m	Milli
µg	Microgram
mg	Milligram
$MgCl_2$	Magnesium chloride

min	Minute
µl	Microlitre
ml	Millilitre
mM	Millimolar
mol	Mole
mRNA	Messenger ribonucleic acid
n	Nano
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NFκB	Nuclear factor kappa B
ng	Nanogram
nm	Nanometer
PBS	Phosphate buffered saline
RLU	Relative light units
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SFM	Serum free media
T	Thymine
TBE	Tris borate-EDTA
TF	Tissue factor
U	Unit

UFH	Unfractionated heparin
UV	Ultra violet
V	Volts
v/v	Volume to volume
VEGF	Vascular endothelial growth factor
w/v	Weight to volume

CHAPTER 1

Introduction

1.0 Introduction

1.1 Cancer-related thrombosis

It has long been established that thrombosis is a common complication observed in cancer patients. The first description of deep vein thrombosis (DVT) in cancer patients was made by Bouillard in 1823 (Bouillard and Bouillaud, 1823). Trousseau later observed that patients with known cancer have an increased propensity to acquire venous thromboembolism (VTE) (Trousseau, 1865). Furthermore, in 1878, Billroth described blood clots and fibrin-like material in tumours during post-mortem examination which suggested a link between clotting and tumour cells (Billroth, 1878). Until recently the mechanisms underlying the association between cancer and thrombosis were unknown. However, it is now believed there are two key mediators of this link: (1) thrombin, whose broad substrate specificity supports a wide variety of cellular effects relevant to tumour growth and metastasis; and (2) tissue factor (TF), initiator of the coagulation cascade, whose presence on nucleated cells confers responsibility for the generation of cell surface thrombin in many pathological situations (Rickles et al., 2003).

Individuals diagnosed with cancer experience more than a four-fold increase in thromboembolic events (Heit et al., 2000). Epidemiological data has shown that cancer patients have a higher risk of developing postoperative DVT, pulmonary embolism (PE) and VTE than those without underlying malignancy (Lee and Levine, 2003). Furthermore, the risk of VTE related mortality in cancer patients is elevated during chemotherapy treatment (Khorana et al., 2007). Despite development of improved anticoagulant strategies for management of thromboembolic disease, thrombosis still represents 9% of cancer related deaths, making it the second most frequent cause of cancer related mortality (Zwicker et al., 2007).

1.2 Haemostasis and Thrombosis

Haemostasis is a process that limits blood loss when blood vessels are damaged. Normal haemostasis is achieved through a series of crucial steps involving vasoconstriction, platelet adhesion and aggregation, and the initiation of the coagulation cascade. Vasoconstriction temporarily decreases the blood flow and pressure of the damaged vessel, while platelets then adhere to exposed collagen at the damaged site and activate platelet aggregation. The exposed collagen and TF then initiate the coagulation cascade, leading to the formation of a fibrin clot which seals the damaged vessel until the tissue can be repaired. Although the process of haemostasis serves to maintain the integrity of the circulation system, the process can go out of balance. Thrombosis, the formation of an obstructive blood clot, is thought to occur when natural anticoagulant mechanisms are impaired or imbalanced during certain physiological conditions and disease states (Colman, 2006).

1.3 The coagulation cascade

The coagulation cascade is divided into three pathways, the intrinsic, extrinsic and common pathways. Coagulation factors circulate as inactive zymogens, then when cleaved by specific enzymes become active serine proteases which in turn activate the next step of the coagulation cascade. A representation of the pathways is shown in figure 1.1

The extrinsic pathway is initiated when circulating FVII binds to the extracellular domain of TF exposed to the vasculature. The TF/FVIIa complex proteolytically cleaves FX which converts it to the active serine protease (FXa). The TF/FVIIa complex is also able to activate FIX which feeds back to the intrinsic pathway. The extrinsic pathway is prevented from progressing further by tissue factor pathway inhibitor (TFPI) which binds to FXa and TF-FVIIa.

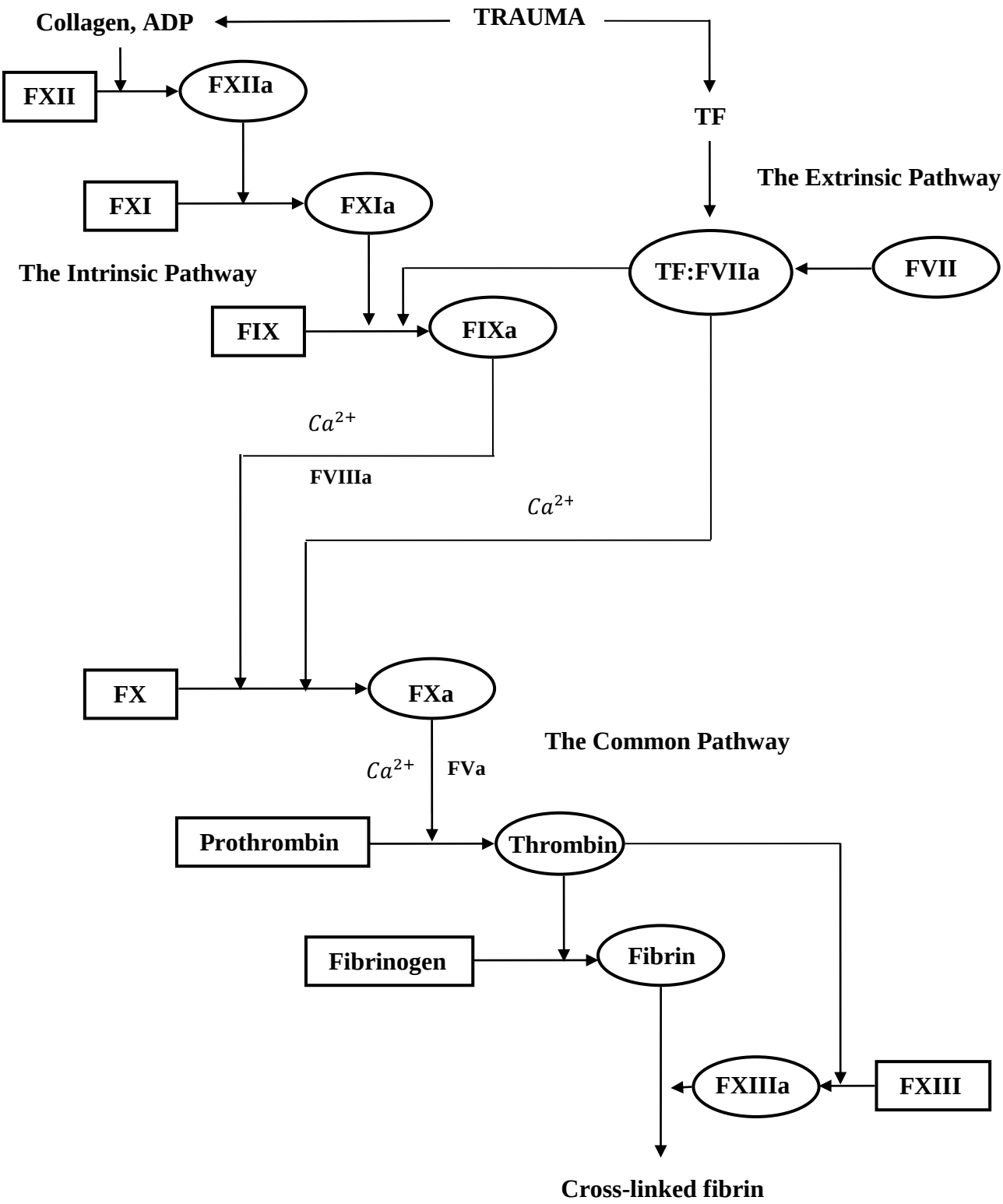

Figure 1. 1 The coagulation cascade.

The extrinsic pathway is activated by TF exposed to the vasculature following injury to blood vessels. As a result of injury to blood vessels, collagen is also exposed to the vasculature which activates the intrinsic pathway. The pathways converge at FX into the common pathway. Activation of FX then leads to the conversion of prothrombin to thrombin which then converts fibrinogen to fibrin resulting in clot formation.

The intrinsic pathway is initiated by the activation of FXII when it comes into contact with negatively charged surfaces of molecules exposed to the vasculature, such as those of collagen (Fig 1.1). The activated FXII then activates circulating FXI which leads to the subsequent activation of FIX. FIXa forms a tenase complex with its co-factor FVIIIa, FX, calcium ions and platelet membrane phospholipids, which results in the activation of FX.

The intrinsic and extrinsic pathways both initiate the common pathway by activation of FX. FXa forms a complex with FVa in the presence of calcium ions and platelet phospholipids. This prothrombinase complex activates the conversion of prothrombin to thrombin. The primary role of thrombin is to induce the formation of fibrin by digesting fibrinogen. Thrombin cleaves fibrinogen resulting in the release of two small peptides from fibrinogens alpha and beta chains. The overall negative charge of the molecule is reduced which results in polymerisation of fibrin monomers. The fibrin clot remains unstable until it is stabilised by thrombin-activated factor XIII, which catalyses cross-linking of fibrin molecules.

1.4 Tissue factor

1.4.1 Structure and Biochemistry

Human tissue factor (TF) or coagulation factor III is a 47 kDa transmembrane glycoprotein composed of 263 amino acids when mature. The human TF gene spans 12.4 kbp, has 6 exons and is located on chromosome 1 (Bluff et al., 2008). TF is a type I integral plasma membrane protein consisting of three domains, the extracellular domain consisting of 219 amino acids, a hydrophobic transmembrane region consisting of 23 amino acids and a 21 amino acid C-terminal intracellular region (Morrissey et al., 1987). The structure of the extracellular portion was refined by crystallographic studies

and was revealed to contain two fibronectin type III modules whose hydrophobic cores merge making a stable platform for FVIIa binding (Muller et al., 1996).

TF is a major regulator of normal haemostasis and thrombosis (Nemerson, 1988). TF is known to be constitutively expressed by astrocytes in the brain, epithelial cells, enveloping organs and body surfaces, adventitial fibroblasts and pericytes, and cardial myocytes in the heart (Osterud and Bjorklid, 2006). In response to various stimuli TF expression and activity can also be induced in endothelial cells and monocytes (Steffel et al., 2006). Moreover, circulating TF has been detected in the bloodstream in the form of microparticles originating from endothelial cells, vascular smooth muscle cells, leukocytes and platelets (Giesen et al., 1999, Schecter et al., 2000, Steffel et al., 2006). In addition to its role in haemostasis and blood coagulation, TF is thought to play a role as a cell signalling receptor as it shares structural homology with the class II cytokine receptor family (Bazan, 1990).

1.4.2 Role of TF in cancer-associated thrombosis

TF expression has been detected in a variety of human tumours, including breast cancer (Ueno et al., 2000), lung cancer (Sawada et al., 1999), leukaemia (Hair et al., 1996), colon cancer (Nakasaki et al., 2002, Seto et al., 2000) and pancreatic cancer (Kakkar et al., 1995b, Ueda et al., 2001). Studies have demonstrated a strong correlation between the expression of TF and the histological grade of malignant tumours (Kakkar et al., 1995a). Furthermore, the expression of TF by cancer cells can directly initiate the coagulation cascade by activating FX (Belting et al., 2005). As a result, TF dependent activation of coagulation has been implicated as playing a central role in cancer-associated thrombosis and metastasis (Fig 1.2) (Kasthuri et al., 2009). Cancer patients with tumours expressing high levels of TF are at an increased risk of developing VTE

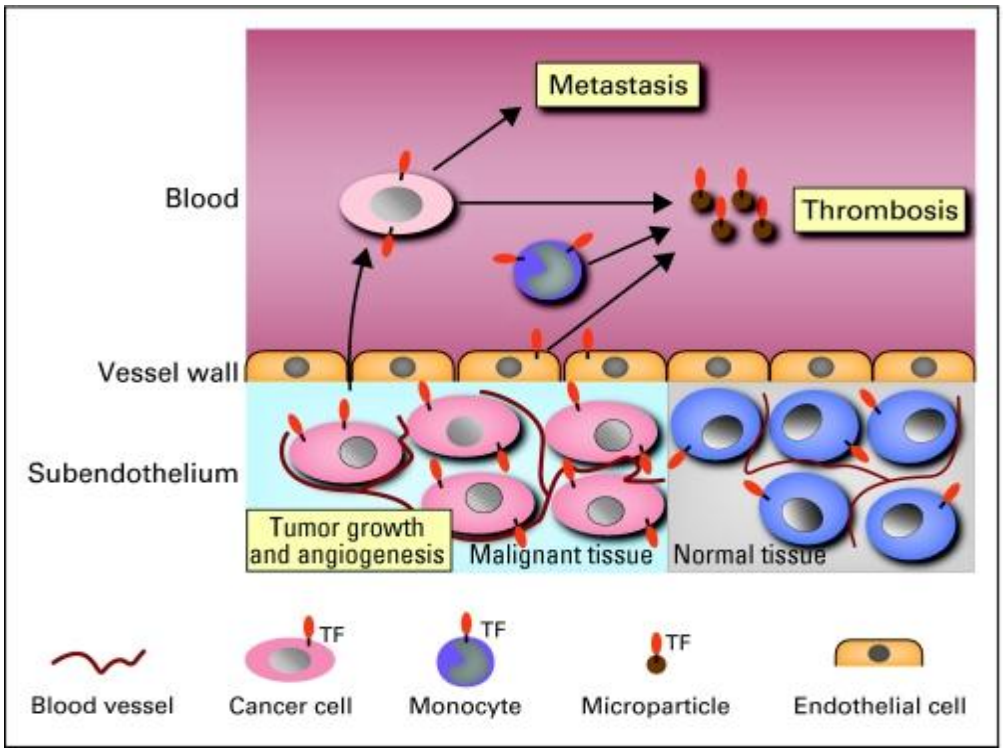

Figure 1. 2 Role of TF in cancer-associated thrombosis

TF is expressed by a variety of different cancer cells which is thought to contribute to tumour growth, angiogenesis and metastasis as well as increased risk of thrombosis in cancer patients. In addition, tumour cells are known to release TF-bearing microparticles into blood circulation which are also thought to contribute to increased risk of thrombosis. The presence of tumour cells may also stimulate monocytes and endothelial cells to elicit a host response which could result in the release of more TF-bearing microparticles (From; Kasthuri *et al*., 2009).

compared to patients with tumours expressing lower levels of TF (Khorana et al., 2007, White et al., 2007). For example, pancreatic cancer patients with low TF expression had a venous thromboembolism rate of 4.5%, which was elevated to 26.3% in patients with high TF expression (Khorana et al., 2007). Similarly, TF expression was found to be significantly higher in ovarian cancer patients with VTE compared to patients without VTE (Uno et al., 2007). In addition to the expression of TF on the surface of tumour cells, many studies have now suggested that tumour cells also release TF-positive microparticles into the bloodstream which contribute to the prothrombotic state observed in cancer patients (Davila et al., 2008, Kasthuri et al., 2009, Tesselaar et al., 2007, Tesselaar et al., 2009, Zwicker et al., 2009).

1.4.3 Circulating TF-bearing microparticles

Circulating microparticles are small (0.1 μm-1 μm) negatively charged vesicles which are emitted from cell membranes into the blood upon activation or during apoptosis. Having long been considered inert debris, microparticles are now known as important cellular effectors involved in crosstalk between cells (Morel et al., 2004). In general, microparticles are derived from circulating cells, such as platelets, leukocytes, and erythrocytes, and cells that compose the vessel wall, mainly endothelial cells, macrophages, and smooth muscle cells (Martinez et al., 2005). However, evidence suggests microparticles are also released by cancer cells (Yu and Rak, 2004, Zwicker, 2010). Microparticles typically express surface proteins and cytoplasmic material derived from the activated parental cell. In particular, the procoagulant activity of microparticles is attributed to the presence of phosphatidylserine and TF on their surface (Morel et al., 2004). Although microparticles are detectable in the blood of normal individuals, the elevated release of TF-bearing microparticles has been described in

various diseases including cardiovascular disease, sepsis, diabetes and cancer (Kasthuri et al., 2009, Morel et al., 2006).

The release of TF-bearing microparticles is reported to be an important link between cancer and thrombosis (Tesselaar et al., 2007). Various studies have demonstrated that microparticle associated tissue factor activity is significantly greater in cancer patients with various malignancies including pancreatic, ovarian, breast and colon compared to normal healthy individuals (Del Conde et al., 2007, Tesselaar et al., 2007). Moreover, several lines of evidence suggest that pathological increases in circulating TF-bearing microparticles are derived from the underlying tumour (Zwicker, 2010). This is supported by *in vitro* studies which have demonstrated various tumour cells shed microparticles containing tissue factor (Dvorak et al., 1983, Yu and Rak, 2004). Suggestions have also been made that tumour cells may also elicit a host response that leads to the induction of TF expression in monocytes and possibly endothelial cells, both of which are known to shed TF-containing microparticles (Kasthuri et al., 2009).

As previously mentioned, various studies have suggested that the level of TF expression correlates with thrombotic risk (Kasthuri et al., 2009, Khorana et al., 2007). Interestingly, recent studies have demonstrated that elevated levels of TF-bearing microparticles are associated with VTE in cancer patients. Cancer patients with VTE have a significantly higher mictroparticle TF activity compared to matched cancer patients without VTE (Tesselaar et al., 2009, Zwicker et al., 2009, Manly et al., 2010). In light of these findings studies are ongoing to evaluate if the level of microparticle TF activity may be a useful biomarker in order to identify cancer patients with increased risk of developing thrombotic complications (Khorana et al., 2008, Tesselaar et al., 2009, Zwicker, 2010).

1.4.4 Regulation of TF expression by NFκB

NFκB is a nuclear protein found in essentially all cell types that was first identified by Sen and Baltimore in 1986 as a transcription factor in B-lymphocytes (Sen and Baltimore, 1986). The NF-κB family of inducible transcription factors is responsible for the induction of a large number of genes that are involved in inflammation, tumour invasion and tumour angiogenesis (Bharti and Aggarwal, 2002). The NF-κB family consists of five dimers which are classified into two functional groups. The first group contains NFκB1 (p50/p105) and NFκB2 (p52/p100) dimers and the second group contains RelA (p65), c-Rel and Rel B dimers. Heterodimers are formed containing different combinations of dimers, the most common heterodimer consists of RelA (p65) and NFκB1 (p50/p105) (Verma et al., 1995). The second group of dimers have a carboxy-terminal transcription domain which is usually required within the Rel/NFκB heterodimer to promote transcription. In most resting cells, NFκB is retained within the cytoplasm bound to the inhibitory IκB protein which blocks the nuclear localisation sequences of NFκB (Baldwin, 1996). NFκB is activated by a variety of stimuli including inflammatory cytokines, lipopolysaccharide and growth factors, which promote the disassociation of IκB through phosphorylation and subsequent degradation by the ubiquitin-dependant pathway (Flossel et al., 1992). The degradation of IκB allows the translocation of NFκB to the nucleus where it binds to NFκB DNA sequences resulting in the activation of gene transcription (Fig 1.3).

NFκB is known to induce the expression of TF in tumour cells (Mackman, 1997). The TF gene contains a promoter region, a segment of DNA that occurs upstream from the gene coding region, which acts as a controlling element for the expression of TF. A 56 bp region between -227 and -172 of the promoter region of the TF gene contains two AP-1 sites and an NFκB site (Mackman et al., 1991) (Fig 1.4). Engagement of the

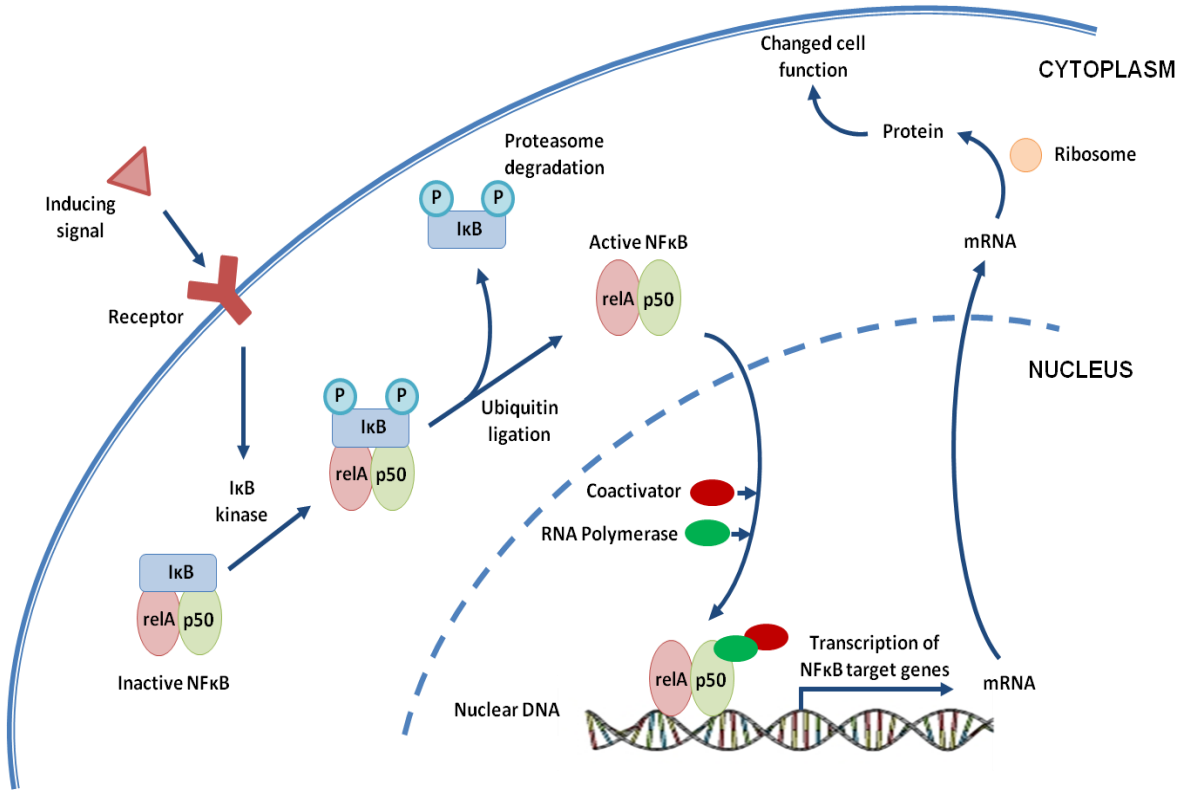

Figure 1. 3 The activation of NFκB

The NFκB heterodimer (Rel A/p50 in diagram) is located in the cytoplasm bound to the inhibitory IκB protein. The NFκB heterodimer is activated in response to stimuli which promote the dissociation of IκB which is phosphorylated and ubiquitation marks it for degradation. This leaves NFκB free to translocate to the cell nucleus where it binds to NFκB DNA binding sequences and activates gene transcription of proteins.

-227 **AP-1** **AP-1** -193

5’ –CGGT**<u>TGAATCA</u>**CTGGGG**<u>TGAGTCA</u>**TCCCTTGCAGG-3’

-192 **NFκB** -172

5’ - GTCC**<u>CGGAGTTTCC</u>**TACCGGG – 3’

Figure 1. 4 <u>Promoter region of the TF gene containing the NFκB binding site</u>

The promoter region of the TF gene contains a 56 bp region between -227 to -172 that contain two AP-1 binding sites and a NFκB binding site that are responsible for increased transcriptional activity of the TF gene (Mackman, 1995, Mackman, 1997). Translocation of an activated NFκB heterodimer to the cell nucleus results in the engagement of the NFκB binding site and induction of TF expression.

NFκB binding site is required to mediate the transcriptional induction of the TF promoter and thus the regulation of TF expression. Over recent years NFκB has been under increased investigation as a potential target for anti-cancer drug development (Garg and Aggarwal, 2002). Increased NFκB activation has been associated with increased inflammation in cancer patients (Pikarsky et al., 2004). Furthermore, studies have suggested that chronic inflammation, via activation of NFκB, generates a tissue environment in which tumour cells of an epithelial cell origin can not only survive but can also stimulate metastasis (Mann and Oakley, 2005).

1.5 Heparin

Heparin, also known as unfractionated heparin (UFH), is a complex carbohydrate belonging to the glycosaminoglycan family and is comprised of mucopolysaccharide chains of various lengths ranging from 5000 to 40000 Da (Verstraete, 1990). It is a natural polyanion composed of repeating disaccharide units of D-glucosamine and uronic acid linked by interglycosidic bonds (Bentolila et al., 2000). Heparin has a high negative charge density due to the amino and hydroxyl groups of the glucosamine units being partially sulphated (Fig 1.5).

The discovery of heparin happened by chance in 1916 by McLean who was looking for procoagulant activity in canine liver cells, but instead found an anticoagulant (Dinis da Gama, 2008). Further investigation has revealed that heparin is a naturally-occurring anticoagulant found in abundance in the liver, spleen, muscle and lungs of most mammals. Furthermore, heparin is found in the secretory granules of mast cells and is released at low levels to prevent the formation of clots within the blood upon injury (Guyton and Hall, 2006). It was not until 1937 that heparin was purified and considered safe and widely available for therapeutic use as an anticoagulant. Pharmaceutical-grade heparin can be extracted from the mucosal tissue of slaughtered animals such as porcine

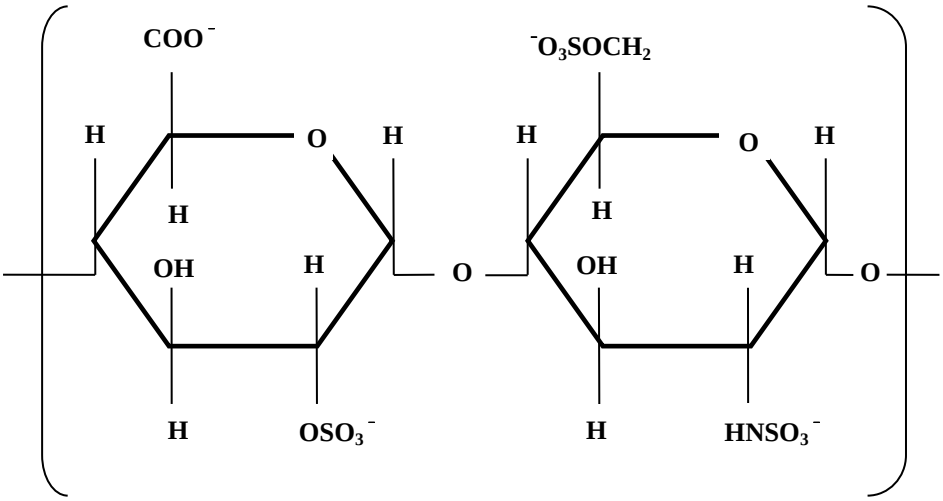

Figure 1. 5 Chemical structure of a repeating unit of unfractionated heparin

The basic disaccharide unit makes up the majority of the polysaccharide; however there are some interruptions in the chain where certain groups are replaced by others. O-sulfonate groups and hydroxyl groups can replace each other, likewise N-acetyl groups can be replaced by N-sulfonate groups or non-substituted amino groups. Adapted from Petitou *et al* (2003).

intestines and bovine lung (Linhardt and Gunay, 1999). Although used primarily as an anticoagulant, because of its high negative charge density heparin can also be used to coat surfaces of medical equipment such as test tubes and renal dialysis machines to prevent coagulation.

1.5.1 The anticoagulant mechanism of heparin

The action mechanism of heparin was not discovered until 1939, when it was suggested that heparin is an indirect anticoagulant requiring a plasma cofactor (Brinkhous et al., 1939). This heparin-cofactor was later named antithrombin (AT) (Abildgaard, 1968). AT is a glycoprotein produced by the liver and is a serine protease inhibitor capable of inactivating enzymes in the coagulation cascade such as thrombin, FXa and FIXa. The arginine-reactive site on the AT molecule is responsible for inhibiting the coagulation enzymes. Heparin binds to the lysine site on AT and produces a conformational change at the arginine-reactive site that increases the action of AT (Rosenberg and Bauer, 1994). Studies revealed that only certain heparin polysaccharide units were able to form complexes with AT and heparin unable to form a complex with AT was considered an inactive form of polysaccharide (Lam et al., 1976). Furthermore, anticoagulant activity was only displayed by the fraction of polysaccharides that were able to form a complex with AT (Petitou et al., 2003). Continued investigation established the existence of an AT-binding site in heparin that was required for it to successfully form a complex with AT. The AT-binding site consists of a specific pentasaccharide sequence containing vital units that are required for high affinity binding of AT (Fig 1.6) (Thunberg et al., 1982). The binding of AT to the pentasaccharide sequence in heparin results in the activation of two mechanisms that increases the inhibition of thrombin and other coagulation factors (Rosenberg and Bauer, 1994). The first mechanism involves heparin serving as a catalytic surface to which AT and thrombin can both bind, forming a

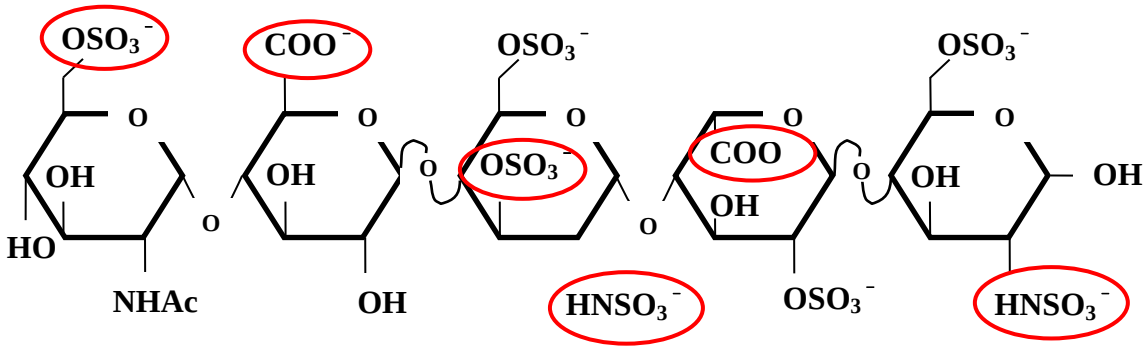

Figure 1. 6 Structure of the pentasaccharide antithrombin binding site of heparin

The groups circled in red play a critical role in heparins interaction with AT, removal of any of the groups would results in decreased ability of heparin to bind to AT and inhibit coagulation. Adapted from Petitou *et al* (2003).

ternary structure that results in the inhibition of thrombin (Li et al., 2004). The second mechanism depends on the conformational change within AT that promotes inhibition of FIXa and FXa. The expulsion of the N-terminal portion of the reactive centre loop (hinge region) from the main beta-sheet A is thought to be responsible for activating the conformational change that inhibits FIXa and FXa (Langdown et al., 2004). Heparin can inhibit many different coagulation enzymes at various points within the coagulation cascade and is also capable of binding endothelial cells and platelets (Hirsh et al., 2001, Rosenberg and Bauer, 1994). In recent years heparin-derived anticoagulants, known as LMWHs, have been developed in order to provide a more specific anticoagulant with reduced hemorrhagic side effects.

1.5.2 Low molecular weight heparin

The use of heparin as an anticoagulant was replaced by LMWH in the 1980's as trials showed that LMWH was a much more effective and reliable anticoagulant. LMWH's are polysulphated glycosaminoglycans that are almost one third the molecular weight of heparin, containing short chains of polysaccharides with an average molecular weight of less than 8000 Da (Hirsh and Raschke, 2004). LMWH can be injected subcutaneously, requires less monitoring, and has an improved efficiency-to-safety ratio compared with heparin (Bounameaux and Goldhaber, 1995). Heparin can be chemically or enzymatically depolymerised to produce LMWH's that vary in pharmacokinetic properties and anticoagulation ability. Commercially available LMWH's differ in structure and molecular weight depending on the method of depolymerisation. For example, deaminative cleavage of heparin with nitrous acid produces dalteparin, which has an average molecular weight of 6000 Da. In contrast, alkaline beta-eliminative cleavage of the benzyl ester of heparin, produces enoxaparin, which has an average molecular weight of 4500 Da (Linhardt and Gunay, 1999).

In comparison to heparin, LMWH's have a decreased ability to inhibit thrombin because they are unable bind to both AT and thrombin simultaneously as they contain short chains. Thrombin must bind adjacent to AT on a flanking sequence in heparin, therefore it requires a heparin chain of more than 18 saccharides to be inactivated (Linhardt and Gunay, 1999, Rosenberg, 1997). However, LMWH's are still able to successfully inactivate FXa activity because that does not require the formation of the ternary complex. FXa interacts with AT bound to the pentasaccharide sequence in LMWH, therefore only short heparin chains are required containing these saccharide units for the inhibition of FXa (Donayre, 1996). FXa lies at the convergence of the extrinsic and extrinsic pathway so by specifically inhibiting FXa, LMWH acts as a more subtle regulator of coagulation (Linhardt and Gunay, 1999, Rosenberg, 1997).

1.6 The potential benefits of LMWH for cancer patients

Clinical trials demonstrated that LMWH reduced the risk of recurrence of deep vein thrombosis (DVT) in cancer patients by 38% compared with UFH (Gould et al., 1999). In addition, LMWH is not only thought to be superior in reducing the risk of thrombotic events but is also linked to increased survival rate in cancer patients (Kakkar and Williamson, 1997). Clinical trials have shown that dalteparin improves the survival rate of cancer patients with advanced malignancy, compared to untreated placebo patients (Kakkar et al., 2004). Examination of the overall patient population in this study showed that dalteparin did not significant increase patient survival compared to patients treated with a placebo. However survival rate was greatly improved when a post-hoc analysis was carried out on of a subset of patients who survived beyond 17 months. In these patients the mean survival time was 43.5 months in the dalteparin group compared to placebo patients whose mean survival time was 24.3 months (Kakkar et al., 2004). The observation of improved survival in cancer patients with advanced metastatic

disease could have future implications on its feasibility in the long term treatment of cancer patients.

In addition to its anticoagulant effect LMWH has been reported to have beneficial effects beyond the control of the hypercoagulable state of cancer patients (Khorana and Fine, 2004). Studies have attributed some of these beneficial effects to the ability of LMWH to suppress TF expression (Gori et al., 1999, Kakkar et al., 2004, Kakkar and Williamson, 1997). Furthermore, recent clinical trials reported that the plasma TF antigen level was reduced in cancer patients treated with dalteparin compared to untreated patients, this was weakly correlated to reduced cellular invasion assessed *in vitro* (Maraveyas et al., 2010). However, the majority of data reporting the influence of LMWH on TF expression has been acquired from *in vivo* measurements carried out in cancer patients during clinical trials. Therefore the mechanism by which LMWH can downregulate TF expression is still unclear.

1.7 Aims of the investigation

The aim of this study is to investigate the influence of LMWH on expression and activity of tissue factor in a variety of cancer cell lines; pancreatic, breast, colocarcinoma, ovarian and melanoma. The study will explore potential mechanisms for down-regulation of tissue factor by LMWH. Finally the study will evaluate the influence of LMWH on cancer cell invasion.

CHAPTER 2

Materials and Methods

2.0 Materials and Methods

2.1 Materials

Active Motif, Rixensart, Belgium
Protease inhibitor cocktail

Affinity Biologicals, Ancaster, Canada
TF-antigen ELISA kit

Ambion/Applied Biosystems, Warrington, UK
MAXIscript®- T7T3 in vitro-transcription kit

American Diagnostica Incorporation, Stamford, USA
Recombinant tissue factor (TF)

Amersham Biosciences, Little Chalfont, UK
GFX PCR DNA and gel band purification kit
Ready-To-Go RT-PCR beads

Applied Biosystems, Nieuwerkerk, Netherlands
PowerSYBR Green RNA-to-C_T 1-Step kit

BD Biosciences, Oxford, UK
T75 (75 cm^2) flasks

BDH Laboratories Supplies, Poole, UK
Magnesium chloride
Sodium acetate
Sodium hydroxide

Berthold Technologies Ltd, Hertfordshire, UK
Junior LB 9509 luminometer
Luminometer tubes

Bio-Rad, Hemel Hempstead, UK
iCycler thermal cycler

BioWhittaker® Lonza, Belgium
Phosphate Buffered Saline (PBS)

Enzyme Research Labs, Swansea, UK
Sheep anti-human TF
Sheep anti-human TF peroxidise conjugated IgG

Eppendorf AG, Cambridge, UK
Microcentrifuge

Gibeo Invitrogen Corporation, Paisley, UK
Lipofectin reagent

OptiMEM reduced serum media

Greiner Bio-One Ltd, Gloucestershire, UK
12- well plates, 24 -well plates

Heraeus Holding GmbH, Hanau, Germany
Heraeus Incubator

IBM UK Ltd, Middlesex, UK
Statistical package for social sciences (SPSS) (PASW Statistics 18.0.3)

International Equipment Company, Bedfordshire, England
Temperature controlled Centrifuge (EC Centra-3RS)

LGC-ATCC, Teddington, UK
Bx-PC-3, LoVo, MDA-MB-231, SKOV-3, A375 cell lines

Promega Corporation, Southampton, UK

Hind III

Luciferase reagent (Luciferin)

Lysis buffer

Recombinant luciferase protein

Tetramethyl Benzidine stabilised substrate for horse radish peroxidase

T4-DNA polymerase

Tris-borate EDTA (TBE) buffer

Wizard® Plus Midipreps – DNA Purification System

R&D systems, Abingdon, UK

Escherichia coli TB-1 strain

Scientific Laboratories Supplies, Hessle, UK

Heidolf Unimax 1010/Inkubator 1000 – Incubated orbital shaker

Sigma Chemical Company Ltd, Poole, UK

Absolute ethanol

Antibiotic/ antimycotic solution (1%)

Basic fibroblast growth factor

Bradford reagent

Calcium chloride

Chloroform

Collagen type IV

DMEM medium

DNase/RNase free water

DMSO

Glutaraldehyde

Isopopanol

Low molecular weight heparin

L-alanyl-L-glutamine (Glutamax)

LB broth powder

Minimum essential medium Eagle (MEM)

Eagles Minimum Essential Medium (EMEM)

PBS tablets

RNase free water

RPMI medium

TRI reagent

Trypsin/EDTA solution

Tween 20

Stratagene, Amsterdam, Netherlands

Pathdetect pNFκB-Luc plasmid

TCS Cellworks Ltd, Clayton, UK

Foetal Calf serum (FCS)

VWR international Ltd, Leicestershire, UK

Boyden chambers (8µm pore size)

Weber Scientific International Ltd, Teddington, UK

Haemocytometer

WPA Lightwave UV/Vis Diode Array, Cambridge, UK

WPA lightwave UV/Vis diode-array spectrophotometer

2.2 Cell culture

All cell culture procedures were carried out under sterile conditions in a class II laminar flow cabinet. Cell lines used were cultured in T75 (75 cm^2) flasks with the appropriate media as follows. The ovarian cancer cell line SKOV-3 in McCoy's 5a medium; the pancreatic cancer cell line BxPC-3 in RPMI-1640 medium; the colocarcinoma cell line LoVo in F-12K media, the breast cancer cell line MDA-MB-231 in Dulbecco's modified eagle's medium (DMEM) and the malignant melanoma cancer cell line A375 in Eagle's minimum essential medium (EMEM). All media were supplemented with 10% (v/v) FCS and 1% (v/v) antibiotic containing penicillin (5 units/ml), streptomycin (5 µg/ml) and amphotericin (25 ng/ml). Cells were incubated at 37°C in a humid environment under 5% (v/v) CO_2. Depending on the growth rate, half the cell medium (3 ml) was replaced with fresh medium every 2-3 days. Cells were subcultured into new flasks when approximately 80% confluent.

2.3 Cell harvesting and sub-culture

The media was removed from the flask and transferred to a 20 ml sample tube for later use. Cells were washed with 8 ml of prewarmed PBS (pH 7.2) to remove all traces of medium. Trypsin/EDTA (5 mg/ml porcine trypsin, 2 mg/ml EDTA) was added to the flask and the cells were then incubated for 3-5 min at 37°C to allow the trypsin to detach the cells from the flask. In order to dislodge the cells from the flask it was tapped firmly. The cell suspension was transferred to the original cell medium in order to neutralise the detrimental effects of trypsin. The cell suspension was centrifuged at 400 *g* for 5 min to pellet out the cells. The media was removed avoiding disruption of the pellet, and the cells were the resuspended in 5 ml of fresh media. To calculate the cell number and density a sample of cell suspension (20 µl) was removed and examined

with a haemocytometer. The number of cells in five 1 mm^2 squares was counted and the number of cells per ml was then calculated using the following equation;

Number of cells per ml = average number of cells counted per mm^2 x dilution factor x 10^4

New cultures were prepared by adding 10^6 cells to fresh T75 flasks with 10 ml of prewarmed complete media.

2.4 Cryopreservation and recovery of cells

Cells were washed in PBS, trypsinised and cell density determined as described in section 2.3. The cells were resuspended in normal media containing 10% DMSO and aliquoted into cryovials at a density of 10^6 cells/tube. The vials were then placed at -70°C in a freezing vessel which enabled a cooling rate of -1°C/min. The cells were then transferred into liquid nitrogen. To recover the frozen cells and start new cultures, the cells were thawed at 37°C and transferred to a fresh T75 flask with 10 ml of prewarmed complete media. The cells were incubated at 37°C in a humid environment under 5% (v/v) CO_2.

2.5 Isolation of total RNA from cell samples using the Tri-Reagent system

The TRI reagent procedure was used to isolate total RNA from cells. In order to prevent contamination and degradation of the RNA all work surfaces were cleaned and RNase-free tubes and filtered pipette tips were used throughout. Cells harvested with trypsin, were lysed in TRI reagent (200 µl) by repeated pipetting and incubated at room temperature for 5 min to ensure complete lysis. Chloroform (40 µl) was added to each tube, samples were vortexed for 15 s and allowed to stand at room temperature for 12-15 min. The samples were centrifuged at 12000 *g* for 15 min and the colourless upper aqueous phase was transferred to a fresh 1.5 ml RNase free tube. The RNA was precipitated by adding isopropanol (100 µl) to each sample which was mixed by vortexing and incubated for 30-60 min at -20°C. The samples were centrifuged for 10

min at 12000 *g* to pellet the RNA. The supernatant was discarded and the pellet washed with 75% (v/v) ethanol (200 µl), vortexed and centrifuged for a further 5 min at 12000 *g*. All the excess ethanol was removed and the RNA pellet was resuspended in RNase free water (60 µl). All RNA samples were stored at -70°C until they were required. To determine the concentration of the RNA samples, the absorption of each sample was measured at 260 nm using a spectrophotometer (See section 2.6).

2.6 Determination of concentration and purity of nucleic acids

The concentration and purity of nucleic acids was determined by measuring the absorption of each sample at 260 nm using a spectrophotometer. The spectrophotometer was calibrated using 54 µl of distilled water in a quartz micro-cuvette. The sample (6 µl) was added to the cuvette, mixed and the absorption was measured at 260 nm. The $A_{260\,nm}$ value was used to determine the concentration of the plasmid DNA/RNA using the following formulae:

Plasmid DNA concentration (µg/ml) = $A_{260\,nm}$ reading × 50 × dilution factor

Plasmid RNA concentration (µg/ml) = $A_{260\,nm}$ reading × 40 × dilution factor

Purity of the DNA/RNA was determined by measuring the 260:280 ratio, a ratio of above 1.8 indicated DNA/RNA of sufficient purity for use.

2.7 Preparation of standard TF mRNA for quantitative real-time RT-PCR

To prepare a TF mRNA standard, full length TF cDNA was cloned into the pT7T3-18 vector (see section 2.13) and used to express target mRNA according to the following method. The plasmid (1 µg) was digested with Sac I (10 units) by incubation at 37°C for 1 h. The digested DNA was then purified using the DNA illustra GFX PCR DNA Purification kit according to manufacturer's instruction. Following purification, the 3'-overhangs were filled in with T4-DNA polymerase (1µl) (9.7 units/ml) which was added to the plasmid (43 µl) and incubated for 1 h at 37°C. The plasmid DNA was then

purified again using the illustra GFX PCR DNA Purification Kit. TF mRNA was transcribed using the MAXIscript®-T7T3 *in vitro*-transcription kit according to manufacturer's instruction. The DNA was destroyed with DNase I (1 µl) for 15 min at 37°C and the mRNA was precipitated and washed with 70% (v/v) ethanol (200 µl). The mRNA was pelleted by centrifugation at 12000 *g* for 5 min and then resuspended in RNase free water (50 µl). The concentration of TF mRNA was determined as described in section 2.6.

The identity of mRNA was confirmed to be that of TF using primers to full-length TF. One-step end-point RT-PCR was carried out using Ready-To-Go RT-PCR beads and 100 ng of standard mRNA. The RT step was carried out at 42°C for 30 min followed by denaturation for 5 min at 95°C. PCR amplification (30 cycles) was performed as follows: 1 min at 95 °C, 1 min at 60 °C, followed by 1 min at 72 °C. The primers used were (TF-forward) 5′-ACCTGGAGACAAACCTCGGAC-3′ and (TF-reverse) 5′-GAGTTCTCCTTCCAGCTCTGC-3′. Products were visualised on a 1.5% (w/v) agarose gel (Ettelaie et al., 2007a, Ettelaie et al., 2008).

2.8 Measurement of TF mRNA expression by quantitative real-time RT-PCR

Cells (2×10^5 / well) in complete media (1 ml) were seeded into 12 well plates and incubated for 24 h at 37°C to allow adherence. The cells were then treated with a range of concentrations of LMWH (0-2000 µg/ml) for 24 h at 37°C, or with LMWH (200 µg/ml) for up to six days. Following incubation the media was discarded, the cells were washed in PBS (1 ml), and harvested using trypsin (300 µl) and repeated pipetting. The cell suspension for each sample was transferred to a 1.5 ml microfuge tube and the cells were pelleted by centrifugation at 12000 rpm for 5 min in a microcentrifuge. The trypsin was discarded and total RNA was isolated from the pelleted cells using the Tri-reagent system as described in section 2.5.

The annealing temperature and number of amplification cycles for RT-PCR were optimised using in vitro transcribed TF mRNA (Research group of Dr Ettelaie). Each RNA sample was amplified with TF primers as well as with β-actin primers as a reference. β-actin is a house-keeping gene expressed in all the cells used. Primers were reconstituted in RNase-free water to give a final concentration of 100 pmol/µl. The primers used were;

TF-forward: 5'-TACAGACAGCCCGGTAGAGTG-3'

TF-reverse: 5'-GAGTTCTCCTTCCAGCTCTGC-3'

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

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

All samples were prepared in triplicate and analysed with both primers using 100 ng of total RNA from each sample tested. A range of TF standards (0.05-10 ng) were set up alongside using previously prepared TF mRNA (see section 2.7). The reaction was carried out on an iCycler thermal cycler at annealing temperatures of 60°C using the PowerSYBR Green RNA-to-C_T Kit according to manufacturer's instructions.

In order to quantify the amount of TF mRNA in each unknown sample a standard curve was prepared for each experiment using a set of TF mRNA standards of known mRNA concentrations (10-0.5 ng). The fold change of the standards was converted to a log10 scale to facilitate comparison, and the Ct values of the standards were plotted against the log10 concentration in a graph (Fig 3.1). The TF mRNA Ct values were normalised against their respective Ct values for β-actin and then the amount of TF mRNA in each unknown sample was calculated from the standard graph using the equation of the trend line. The log10 concentration of TF mRNA in each sample was then converted back into TF mRNA (ng). This value is the amount of TF mRNA expressed by the number of cells in each well (2×10^5 / well). Therefore, in order to express the data as TF mRNA

(ng) per 1 million cells, the amount of TF mRNA for each sample was divided by the number of cells in each well (2×10^5 / well), and then multiplied by 1 million.

2.9 Estimation of protein concentration in cell samples using a Bradford assay

The concentration of total protein from lysate samples (see section 2.10) was determined using Bradford protein reagent. Each cell lysate (10 µl) was diluted 1:10 with distilled water (90 µl). A range of standards were prepared of known protein concentration (0, 10, 50, 100, 150 µg/ml) by serial dilution of BSA in distilled water. The standards and samples (100 µl) were placed into separate 1 ml plastic cuvettes. Bradford's reagent was prepared by diluting the stock reagent with distilled water (1:2 v/v). The diluted Bradford's reagent (900 µl) was added to each cuvette and the samples were incubated at room temperature for 10 min. The absorption of each sample was measured at 595 nm using a spectrophotometer. A standard curve was prepared using the absorption values of the standards (Fig 2.1). The amount of protein in each sample was calculated by comparison with the standard curve and the volume of sample containing 20 µg of protein was calculated for ELISA.

2.10 Measurement of tissue factor antigen using ELISA

Cells (2×10^5 / well) in complete media (1 ml) were seeded into 12 well plates and incubated for 24 h at 37°C to allow adherence. The cells were then treated with a range of concentrations of LMWH (0-2000 µg/ml) for 24 h at 37°C, or with LMWH (200 µg/ml) for up to six days. The cells were lysed in 150 µl of lysis buffer (diluted 1:4 with distilled water), 10 mM DTT and in the presence of 1% (v/v) protease inhibitor cocktail by repeated pipetting. The concentration of total protein in each sample was determined using the Bradford assay as described in section 2.9. Expression of total cellular TF antigen was measured using a TF-antigen ELISA kit (Affinity Biologicals, Canada). Capture antibody (Sheep anti-human Tissue Factor (TF)) was diluted 1/100 in coating

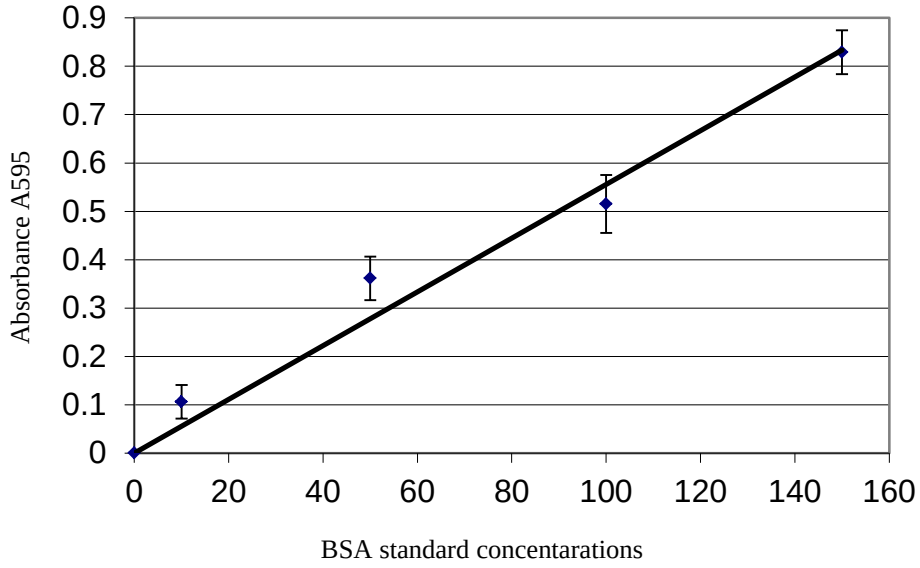

Figure 2. 1 Standard curve for Bradford assay

A range of standards were prepared of known protein concentration (0 -150 µg/ml) by serial dilution of BSA in distilled water. The standards (100 µl) were placed into separate plastic cuvette in duplicate and diluted with Bradford reagent (900 µl). The samples were incubated at room temperature for 10 min and the absorption of each sample was measured at 595 nm using a spectrophotometer. The data represents a typical standard curve which was prepared for each Bradford assay using duplicates of each standard.

buffer (50 mM Carbonate – 0.16% Na_2CO_3, 0.3% $NaHCO_3$, in distilled water, pH 9.6), added (100 µl) to wells on a 96 well microplate and incubated for 24 h at 4°C to allow adherence. Following incubation, the coating buffer was discarded and the wells were blocked with 150 mM PBS (pH 7.4), containing 1% (w/v) BSA for 90 min at room temperature. The blocking buffer was then discarded and the wells were washed four times with PBS-Tween (50 ml PBS, 50 µl Tween-20). A set of standards was prepared by serial dilution of recombinant TF (50, 25, 12.5, 6.25, and 3.13 ng/ml). Standards and 20 µg of protein from each sample were applied to the plate in duplicate and the contents of each well was made up to 100 µl with sample dilutent (2.4% HEPES, 0.6% NaCl, 0.1% Triton X-100, in distilled water, pH 7.2). The plate was incubated for 1 h at room temperature and then washed carefully four times with PBS-Tween (200 µl). The detection antibody (Sheep anti-human TF, peroxidise conjugated IgG) was diluted 1/150 in conjugate buffer (2.4% HEPES, 0.6% NaCl, 0.1% Triton X-100, 1% BSA, in distilled water, pH 7.2), added (100 µl) to each well and incubated for 1 h at room temperature. The plate was washed four times with PBS-Tween (200 µl) and developed by adding HRP substrate (100 µl) to each well. The colour was allowed to develop for 15-20 min in the dark with shaking. The reaction was quenched by addition of 0.5 M H_2SO_4 (50 µl) to each well. Absorption of each well was measured at 450nm using a plate reader. The quantity of TF antigen was determined from the standard curve prepared with recombinant TF. Data were expressed as TF antigen (ng) per 1 million cells to normalise the comparisons between each set of experiments.

2.11 Measurement of tissue factor activity using two-stage chromogenic assay

A two-stage chromogenic assay was used to measure cell surface TF activity following treatment of cells with LMWH. The assay is based on quantifying the activity of generated thrombin. The generated thrombin liberates the p-nitroanilide group of the

chromogenic substrate which results in colour production. Thrombin formation is initiated by the activation of the coagulation cascade by TF. Therefore, the greater the TF activity the greater the thrombin generation which results in a more intense colour being produced, as previously described (Ettelaie et al., 2008). Cells (2×10^5) in complete media (100 µl) were seeded out into 96 well plates and incubated for 24 h at 37°C to allow adherence. The cells were then treated with a range of concentrations of LMWH (0-2000 µg/ml) for 24 h at 37°C, or with LMWH (200 µg/ml) for up to six days. The media was discarded and the cells were resuspended in PBS (20 µl) and incubated with 5 mg/ml barium absorbed plasma solution (20 µl) and 0.05 M Tris-HCl pH 7.2 (40 µl) for 10 min at 37°C. The reaction was initiated by the addition of 25 mM $CaCl_2$ (20 µl). After 20 min the generation of thrombin was quenched by the addition of buffered substrate-EDTA (0.5 M Tris-HCl pH 7.2, 25mM EDTA) containing 1 mM BIOPHEN CS-01(81) thrombin chromogenic substrate. The samples were incubated for a further 20 min at 37°C and the reaction was stopped using 20% (v/v) acetic acid (50 µl). Standards were prepared alongside by making serial dilutions of recombinant TF made from a stock solution (0.5 µg/ml) which was assumed to contain 1000 U/pmol. The absorption of each sample was measured at 405 nm on a plate reader and converted to the equivalent TF activity using the standard curve prepared with recombinant TF. Data were expressed as TF activity (units) per 1 million cells to normalise the comparisons between each set of experiments.

2.12 Preparation of LB broth and propagation of Escherichia coli TB-1

LB broth powder (12.5 g) was dissolved in de-ionised water (500 ml) and autoclaved before use. *E.coli* TB-1 cells were propagated in LB broth (100 ml) with gentle agitation overnight at 37°C. The *E.coli* TB-1 were divided into aliquots (1 ml) and stored at -70ºC until required.

2.13 Transformation of competent bacterial cells

E.coli TB-1 cells were transformed with plasmid DNA (pNFκB-Luc or pT7T3-18) (Stratagene, Netherlands). Competent cells were removed from storage at -70ºC and kept on ice. Plasmid DNA (20 ng) was added to 100 µl aliquots of competent cells and mixed gently, no plasmid DNA was added to the negative control. The cells were incubated on ice for 30 min, heat shocked at exactly 42ºC for 45-50 sec, and immediately returned to ice for a further 2 min. LB broth (900 µl) was added to each tube and the cells were incubated for 1.5 h at 37ºC with shaking at 150 rpm to allow the plasmid to express encoded antibiotic resistant genes.

2.14 Selection of transformed bacteria

Agar/antibiotic plates were prepared by dissolving LB agar powder (3.5 g) in de-ionised water (100 ml). The medium was autoclaved and allowed to cool to 50 ºC before adding the appropriate antibiotic (ampicillin, 100 µg/ml). Approximately 20 ml of LB/antibiotic agar was poured into separate Petri dishes and allowed to set. Plates were sealed and stored at 4ºC for up to 1 month. In order to select transformed bacteria cells, bacterial suspension (50 µl) was evenly distributed on antibiotic-LB agar plates using a sterile glass spreading rod. Plates were incubated overnight at 37ºC. Following incubation, colonies were selected and removed from the plates using a sterile loop and transferred to 10 ml of LB broth. The bacteria were incubated at 37 ºC for 24 h.

2.15 Isolation of plasmid DNA

Plasmid DNA was isolated from the bacterial cells using the Wizard® Plus Midipreps DNA purification systems as follows. The bacterial cell suspension was cooled and centrifuged at 2500 *g* for 25 min. The supernatant was discarded and the bacteria were resuspended in resuspension solution (50 mM Tris-HCl pH7.5, 10 mM EDTA). Cell lysis solution (0.2 M NaOH, 1% (w/v) SDS) was added and mixed by inverting the

tube. Neutralisation solution (1.32 M potassium acetate pH 4.8) was added, mixed by gentle inversion and the lysate was centrifuged at 12000 *g* for 20 min to pellet the cell debris. The supernatant containing the plasmid DNA was decanted and DNA purification resin (8 ml) was added to the supernatant and inverted to mix. The solution was transferred to a DNA binding column connected to a vacuum. Once all the solution had cleared through the column the retained resin was washed with column wash solution (80 mM potassium acetate, 8.3 mM Tris-HCl pH 7.5, 40 µM EDTA, 55% (v/v) ethanol) and cleared under vacuum. The column was placed into a 1.5 ml microcentrifuge tube and centrifuged at 12000 *g* for 20 min to remove excess wash buffer. The column was then placed into a fresh 1.5 ml microcentrifuge tube and pre-heated (65-70ºC) DNase-free water (200 µl) was added to the column. The plasmid was eluted from the column by centrifugation at 12000 *g* for 2 min.

The plasmid DNA (200 µl) was precipitated by adding 3M sodium acetate (200 µl) and 70% (v/v) ethanol (800 µl) and incubated at -20°C for 20 min. The sample was then centrifuged at 12000 g for 20 min to pellet out the plasmid DNA. The supernatant was then discarded and the pellet washed with 70% (v/v) ethanol (100-200 µl) and centrifuged a further 5 min at 12000 g. The ethanol was discarded and the pellet was dried and resuspended in DNase-free water (50 µl). The plasmid DNA was stored at -20°C until required.

2.16 Transfection of cancer cell lines with plasmid DNA using lipofectin

Lipofectin is a lipid based reagent which encapsulates plasmid DNA allowing it to enter cells and be released into the cytoplasm. Prior to transfection cells (2×10^5 / well) were seeded out into 12 well plates in complete media (1 ml) and incubated for 24 h at 37°C. The cells were washed in PBS (1 ml) and pre-adapted to OptiMEM reduced serum media (800 µl) for 1 h prior to transfection. For each transfection, solution A was

prepared by diluting plasmid DNA (pNFκB-Luc) (1 µg) in OptiMEM media (100 µl). Solution B was prepared by diluting Lipofectin reagent (10 µl) in OptiMEM media (100 µl). Both solutions were incubated at room temperature for 30-45 min and the two solutions were then combined and incubated for a further 15 min at room temperature. The lipid-DNA complex (200 µl) was then added to each well and incubated for 6 h at 37°C. Following transfection all the media was removed from each well and the cells were washed in PBS (1 ml) and complete media (1 ml) was added to each well. The cells were incubated for 24 h at 37ºC before treatment with different concentrations of LMWH (0-2000 µg/ml).

2.17 Luciferase Assay

A luciferase assay was used to determine transcription activation of cells transfected with a luciferase reporter plasmid. Luciferase activity was determined by addition of luciferase substrate (luciferin) to cell lysates as described below. The ATP-dependant reaction, in which luciferase converts luciferin to oxyluciferin, produces light, which is measured and used to determine transcription factor activity in the samples.

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow[Mg^{2+}]{} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \textbf{LIGHT}$$

All the media was removed and the cells were washed with PBS (1 ml). Lysis buffer (150 µl) diluted 1: 4 with distilled water was added to each well and incubated at room temperature for 1-2 min. The cells were lysed by repeated pipetting and each sample was transferred to fresh 1.5 ml microfuge tubes. The lysed samples were centrifuged for 5 min at 12000 rpm in a microcentrifuge to pellet the cell debris. The supernatant from each sample was then transferred to a fresh 1.5 ml microfuge tube and kept on ice. The luciferase substrate was thawed and kept in the dark on ice. For each sample the luciferase substrate (100 µl) was added to a luminometer tube, followed by cell lysate (20 µl). This was then mixed and the luciferase activity of each sample was measured

over 5 min using a luminometer. The level of luciferase activity measured in relative light units (RLU). Recombinant luciferase protein was used to determine the highest and lowest levels of RLU that were accurate and also to determine the optimum read time on the luminometer (See section 3.4).

2.18 Investigating the influence of NFκB activity on TF expression

Cells (2×10^5) in complete media (100 µl) were seeded out into 96 well plates and incubated for 24 h at 37°C to allow adherence. The cells were treated with the NFκB inhibitor pyrrolidinedithiocarbamate ammonium (10 µM) alongside an untreated set of cells for 24 h. The cells were lysed in 150 µl of lysis buffer (diluted 1:4 with distilled water), 10 mM DTT and in the presence of 1% (v/v) protease inhibitor cocktail by repeated pipetting. A Bradford assay was used to calculate the amount of protein in each sample (see Section 2.9), and the expression of TF antigen was measured using a TF-specific ELISA (see Section 2.10).

2.19 Investigating the effect of LMWH on TF antigen expression in cancer cell lines supplemented with growth factors

The influence of growth factors, (EGF, VEGF and bFGF) on TF expression in all cell lines in the presence and of absence of LMWH was investigated using a TF-specific ELISA (see section 2.13). Cells (2×10^5) in complete media (1 ml) were seeded out into 24 well plates, and incubated at 37°C for 24 h. The cells were supplemented with a range of concentrations of either EGF (0-10 ng/ml), VEGF (0-4 ng/ml) or bFGF (0-20 ng/ml), in the presence or absence of LMWH (200 µg/ml), and incubated for 24 h at 37°C. The cells were lysed and protein content of each was estimated using a Bradford assay (Section 2.9). The amount of TF antigen was determined by ELISA (Section 2.10) and the concentration of TF antigen determined from the standard curve prepared alongside with recombinant TF.

2.20 Influence of LMWH on cell invasion using Boyden chambers

The influence of LMWH on cell invasion was measured using an assay based on the ability of cells to permeate a collagen barrier in a Boyden chamber. Boyden chambers with a 8 µm pore size were placed into wells on a 24-well plate and coated with collagen type IV (1 mg/ml) overnight at 37°C. The excess collagen was removed and cells (5×10^4) in media (250 µl) were seeded out into the upper compartment of the Boyden chambers. The cells were treated immediately with a range of concentrations of LMWH (0, 20 and 2000µg/ml). Complete media (250 µl) containing bFGF (5 µg/ml) was added to the lower compartment and the cells were incubated at 37°C for 24 h to allow cells to migrate through the collagen barrier (Fig 2.2). Following incubation the media from the upper and lower compartment of each Boyden chamber was removed and discarded. The cells on the upper side of the filter were removed using a cotton wool swab and the empty Boyden chambers were placed back into the wells. The cells were fixed by adding 3% (w/v) glutaraldehyde (150 µl) to the lower compartment and incubation at room temperature for 15 min. The glutaraldehyde was discarded and the cells were washed with PBS before staining. Crystal violet (150 µl) was added to lower compartment of each well and left to incubate at room temperature for a further 20 min. The crystal violet was then removed and the cells were washed three times with PBS to remove any excess stain. The stained cells were agitated at room temperature for 15 min with prewarmed 1% (w/v) SDS until the cell-associated crystal violet was released by the cells. The samples (350 µl) from each well were transferred to individual 1 ml plastic cuvettes and diluted with distilled water (400 µl). The absorption of each sample was measured at 595 nm using a spectrophotometer and converted to cell number from a standard curve. The standard curve was prepared by placing known numbers of cells out into a 24 well plate (5000-70000 cell/well) and incubating at 37°C for 1 h before repeating the procedure as described above.

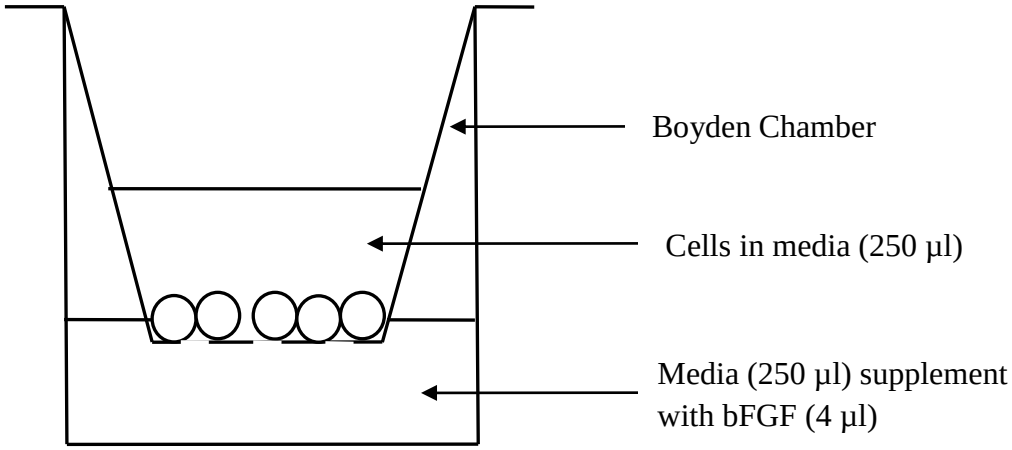

Figure 2. 2 Schematic diagram of a Boyden chamber set up for an invasion assay

Boyden chambers with a 8 µm pore size were placed into wells on a 24-well plate and coated with collagen type IV (1 mg/ml) overnight at 37°C. Cells (5×10^4) were seeded out into the upper compartment of the Boyden chambers in complete media. The cells were treated immediately with a range of concentrations of LMWH (0, 20 and 2000µg/ml). Complete media supplemented with bFGF (5 µg/ml) was added to the lower compartment and the cells were incubated at 37°C for 24 h to allow cells to migrate through the collagen barrier.

2.21 Statistical analysis

All the data values are the mean values of the experiments with the derived standard error of mean. Statistical analysis of the results was carried out using the Statistical Package for Social Sciences (SPSS) version PASW Statistics 18.0.3. Variance of data was analysed and significance determined using one-way ANOVA, p values < 0.05 were taken as significant.

CHAPTER 3

Results

3.0 Results

3.1 Influence of LMWH on cellular TF expression

The expression of TF mRNA was examined by RT-PCR. The RT-PCR efficiency was consistently greater than 83% with an optimal primer concentration of 100 nM. The amplification of the standard TF mRNA was linear over a range of 0.05-10 ng, the correlation coefficient was calculated as 0.989, and therefore the minimum sensitivity of the reaction was assumed to be 0.05 ng of TF mRNA (Fig 3.1).

Baseline expression of TF mRNA was highest in LoVo cells (10.7 ± 3.0 ng/10^6 cells), similar in MDA-MD-231 (8.67 ± 2.8 ng/10^6 cells) and A375 cells (8.6 ± 0.3 ng/10^6 cells), and lowest in BxPC-3 (7.9 ± 1.0 ng/10^6 cells) an SKOV-3 cells (7.7 ± 0.1 ng/10^6 cells) (Fig 3.2). In general, treatment of all the cell lines with increasing concentrations of LMWH (0-2000 µg/ml) resulted in a dose dependent decreased in TF mRNA expression, with LMWH becoming most effective at different concentrations in each cell line. After 24 h a significant ($p<0.05$) decrease in TF mRNA expression, was observed in MDA-MB-231 cells treated with 20 µg/ml or higher concentrations of LMWH. Although suppression of TF mRNA was also observed in BxPC-3 and LoVo cells treated with 20 µg/ml of LMWH, significant ($p<0.05$) downregulation of TF mRNA expression was achieved in these cell lines at LMWH concentrations of 200 µg/ml and above. In addition, significant ($p<0.05$) downregulation of TF mRNA in A375 and SKOV-3 cells was observed at concentrations of LMWH of 200 µg/ml or higher. At the highest concentration of LMWH (2000 µg/ml) the expression of TF mRNA in LoVo and MDA-MB-231 cells was reduced to 2.1% and 0% respectively, of the untreated control. In contrast, at the highest concentration of LMWH (2000 µg/ml) the expression of TF mRNA in SKOV-3 and A375 cells was not reduced to below 60 % of the untreated control. Over a period of six days LMWH (200 µg/ml) reduced the

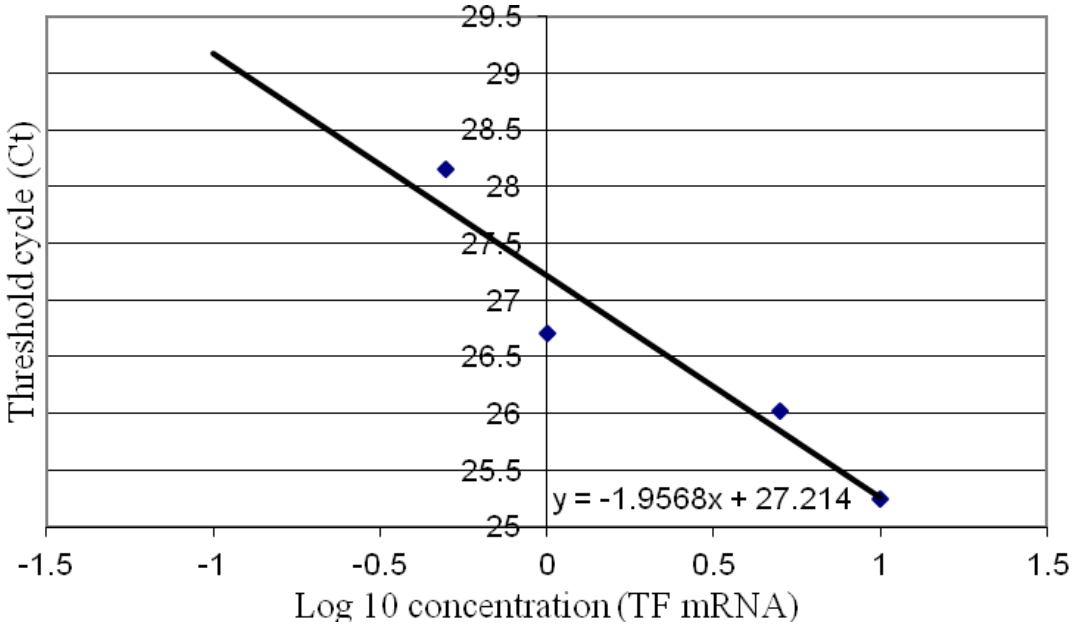

Figure 3. 1 Real-time RT-PCR Standard curve

The real-time RT-PCR standard curve for TF mRNA was obtained at annealing temperatures of 60°C with the PowerSYBR Green RNA-to-C_T Kit using a range of TF standards (0.05-10 ng) which were prepared as described in section 2.7. The Ct values were plotted against log of TF mRNA concentration. The correlation coefficient was calculated to be 0.989 and the PCR efficiency was consistently greater than 83%. The standard curve was used to accurately quantify the amount of TF mRNA in each experimental sample.

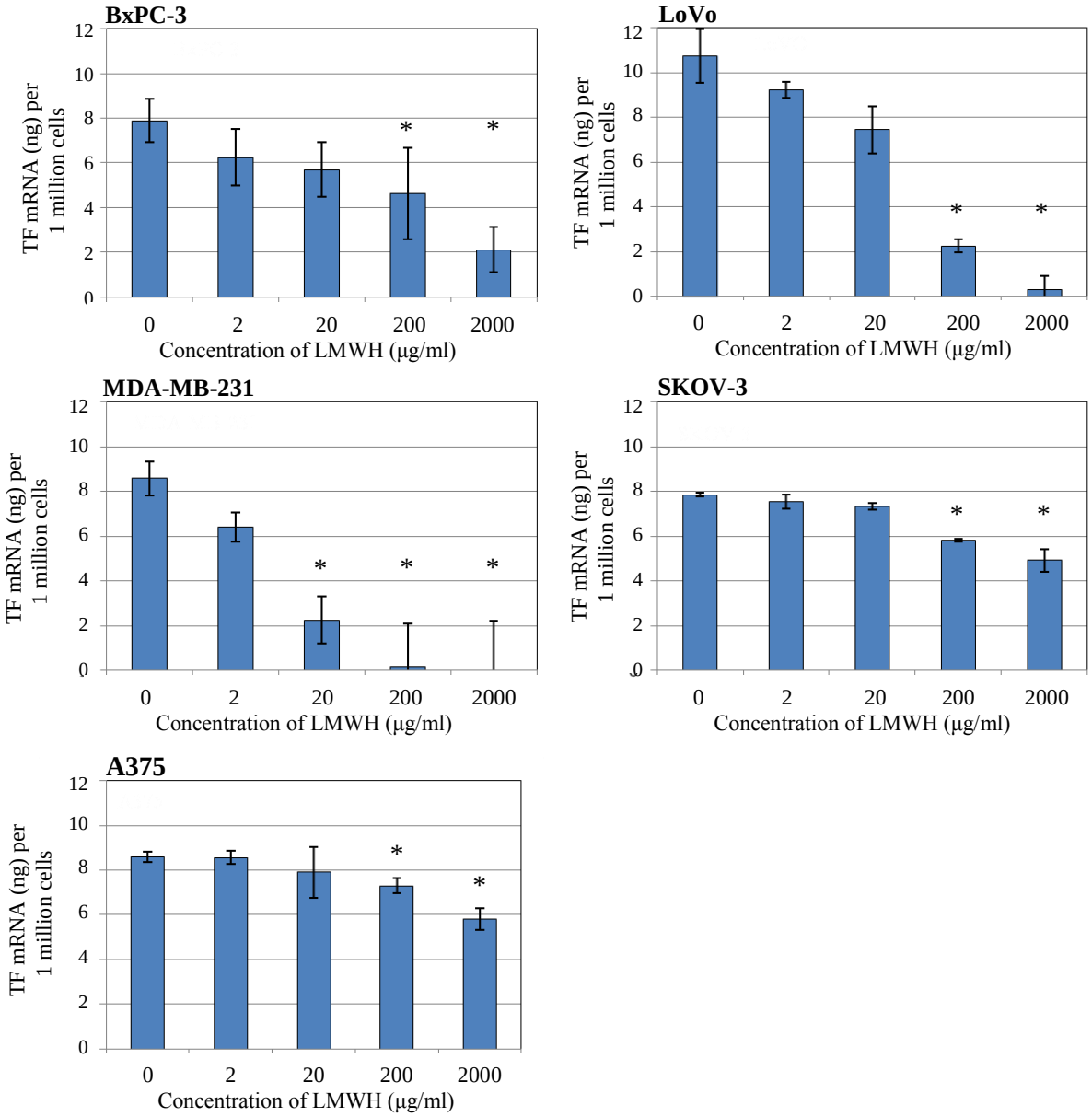

Figure 3. 2 The influence of LMWH on TF mRNA expression

Sets of cells (2×10^5) were treated with a range of LMWH concentrations (0-2000µg/ml) for 24 h. Total RNA was isolated from the cells and the expression of TF mRNA determined by real-time quantitative RT-PCR using specific TF primers. The data were normalised against β-actin mRNA in each sample and the quantity of RNA was determined using a standard curve prepared by amplification of *in vitro*-transcribed TF mRNA. Data represents the mean of three experiments each measured in triplicate (*=$p<0.05$ vs. respective untreated sample). Data published in Ettelaie *et al* (2011).

expression of TF mRNA at different rates in each cell line (Fig 3.3). In LoVo and MDA-MB-231 cells the expression of TF mRNA was rapidly depleted after one day of treatment with LMWH and remained below 1% of the untreated control for the period of six days. In comparison, following treatment with LMWH the expression of TF mRNA gradually decreased in BxPC-3, SKOV-3 and A375 cells. However, after six days of treatment with LMWH, TF mRNA expression was reduced to below 10% of the untreated control in these cell lines (Fig 3.3).

3.2 Influence of LMWH on the expression of TF antigen

The expression of TF antigen was then measured using a TF-specific ELISA. The incubation of all cell lines with a range of concentrations of LMWH (0-2000 µg/ml), for 24 h, resulted in a concentration-dependent decrease in TF antigen. A significant ($p<0.05$) reduction in detectable total TF antigen was observed in SKOV-3 cells treated with concentrations of 2 µg/ml or higher of LMWH (Fig 3.4). The total TF antigen was significantly ($p<0.05$) reduced in BxPC-3 and MDA-MB-231 cells treated with LMWH at concentrations of 20 µg/ml, and above. Furthermore, a significant ($p<0.05$) decrease in detectable total TF antigen in LoVo and A375 cells was achievable at the highest concentrations of LMWH (200-2000 µg/ml). At the highest concentration of LMWH (2000 µg/ml) the level of TF antigen in SKOV-3 and MDA-MB-231 cells was diminished to 13.4% and 25.0 % of the untreated control respectively. In contrast, the total expression of TF antigen in Bx-PC-3, LoVo and A375 cells did not drop below 60% of the untreated control in any of these cell lines. Over a period of six days of treatment with LMWH (200 µg/ml) the level of TF antigen was depleted at different rates in each cell line (Fig 3.5). The prolonged treatment of Bx-PC-3, LoVo and A375 with LMWH over a period of six days resulted in a gradual reduction in the level of TF antigen. However, after six days of treatment with LMWH the level of TF antigen was

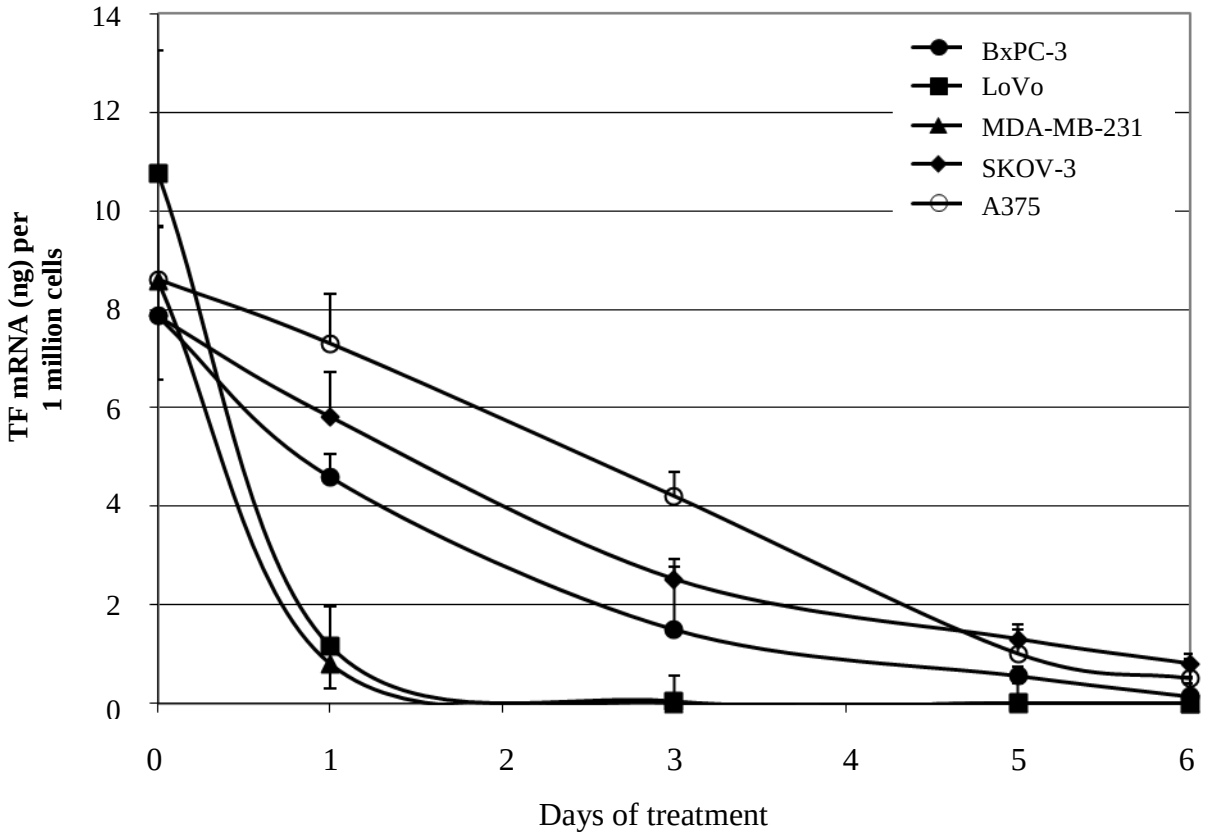

Figure 3. 3 Time-course analysis of the influence of LMWH on TF mRNA expression

Sets of cells (2×10^5) were incubated with LMWH (200 µg/ml) over a period of six days, "0" represents the untreated control samples. Total RNA was isolated from the cells and the expression of TF mRNA determined by real-time quantitative RT-PCR using specific TF primers. The data were normalised against β-actin mRNA in each sample and the quantity of RNA was determined using a standard curve prepared by amplification of *in vitro*-transcribed TF mRNA. Data represents the mean of three experiments each measured in triplicate (*=$p<0.05$ vs. respective untreated sample). Data published in Ettelaie *et al* (2011).

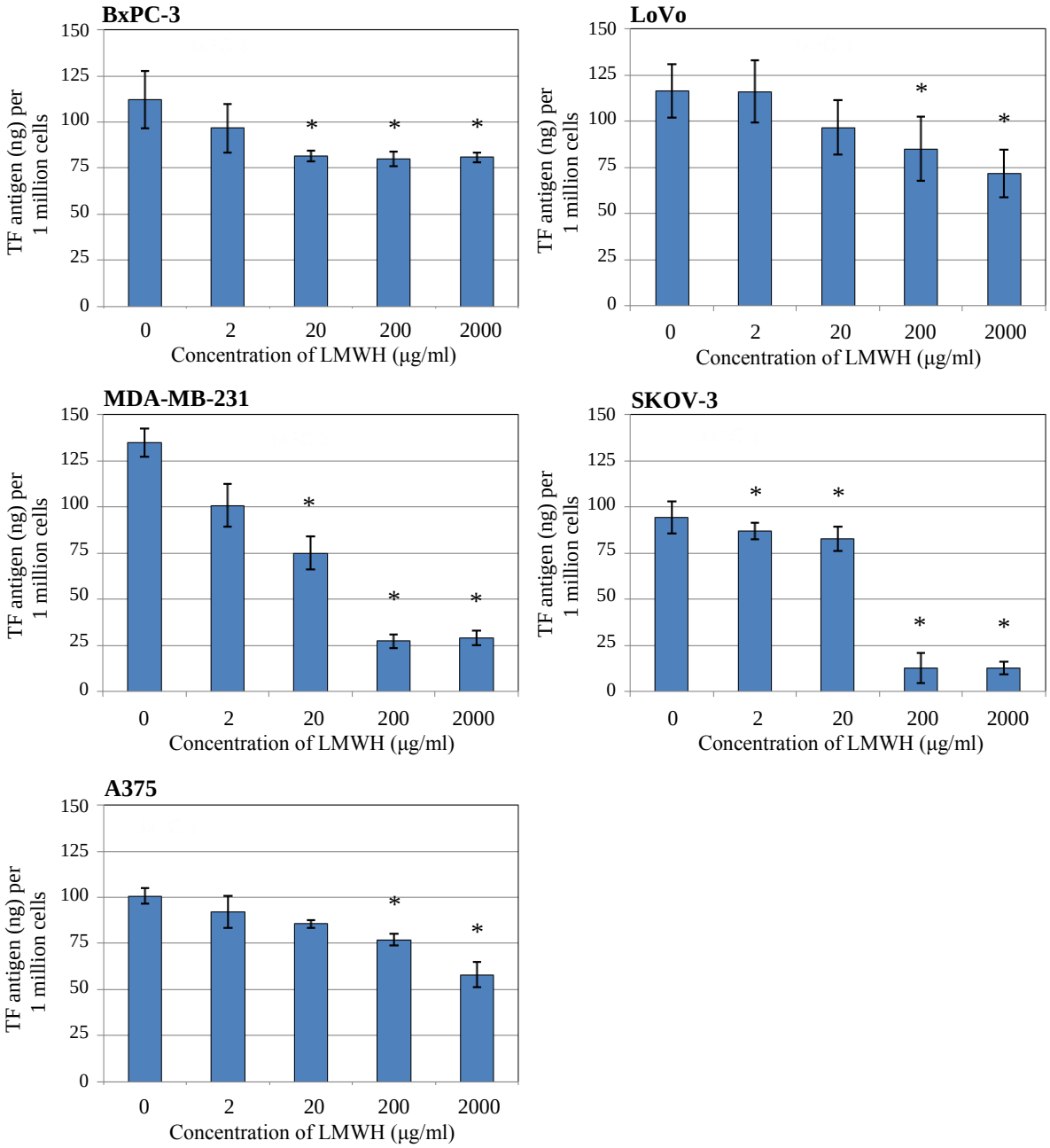

Figure 3. 4 The influence of LMWH on the expression of TF antigen

Sets of cells (2×10^5) were treated with a range of LMWH concentrations (0-2000 µg/ml) for 24 hours. The cells were lysed and the amount of protein per sample was estimated using a Bradford assay. The total TF antigen was determined by ELISA and the TF concentration in each sample was determined using the standard curve prepared with recombinant TF. Data represents the average of four experiments measured in duplicate (*=$p<0.05$ vs. untreated sample). Data published in Ettelaie *et al* (2011).

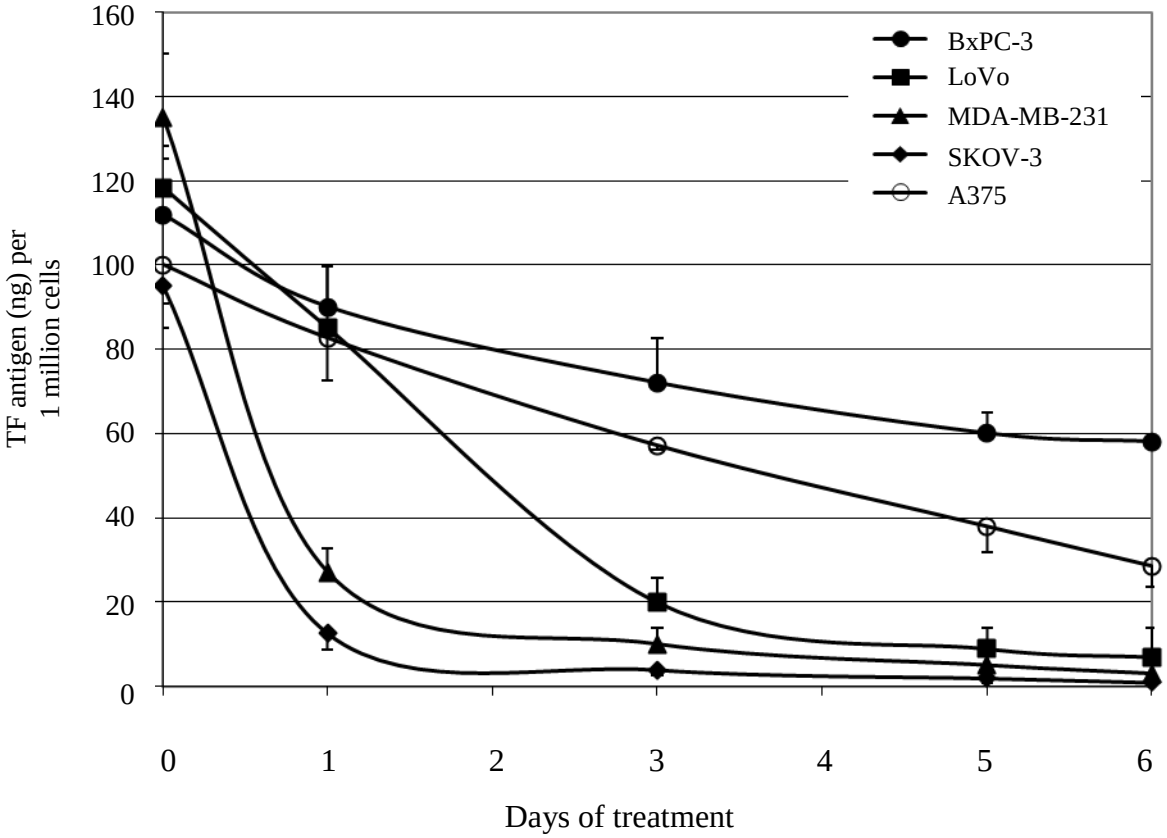

Figure 3. 5 Time-course analysis of the influence of LMWH on expression of TF antigen

Sets of cells (2×10^5) were incubated with LMWH (200µg/ml) over a period of six days, "0" represents the untreated control samples. The cells were lysed periodically and the expression of TF protein was measured using ELISA. The TF concentration in each sample was determined from a standard curve prepared alongside. Data represents the average of three experiments measured in duplicate (*=$p<0.05$ vs. untreated sample).

only reduced to 51.9% and 28.6% of the untreated control in BxPC-3 and A375 cells respectively, compared to below 6% of the untreated control in all other cell lines. The level of TF antigen remained unaltered over six days in untreated cells (data not shown).

3.3 Influence of LMWH on cellular TF activity

Cell surface TF activity in each sample was measured using a two-stage chromogenic assay. A reduction in detectable cell surface TF activity was observed in all cell lines following treatment with LMWH. Basal cell surface TF activity in untreated cells was highest in LoVo cells (432 ± 35 units of TF per 10^6 cells), and lowest in SKOV-3 cells (207 ± 11 units of TF per 10^6 cells) (Fig 3.6). Incubation of MDA-MB-231 cells with 20 µg/ml or higher concentrations of LMWH resulted in a significant ($p<0.05$) reduction of cell surface TF activity. A significant ($p<0.05$) reduction in cell surface TF activity was achieved in BxPC-3, LoVo, SKOV-3 and A375 cells at LMWH concentrations of 200 µg/ml or higher. LMWH reduced the detectable cell surface TF activity in a concentration dependant manner. At the highest concentration of LMWH (2000 µg/ml) the greatest reduction in cell surface TF activity was observed in SKOV-3 cells which was diminished to approximately 48% of the untreated control. LoVo and MDA-MB-231 cell surface TF activity was reduced to approximately 55% and 59%, of the untreated control, when treated with the highest concentration of LMWH (2000 µg/ml). However, cell surface TF activity did not drop below 65% of the untreated control in BxPC-3 and A375 cells, even at the highest concentration of LMWH (2000 µg/ml).

The reduction in TF activity was reduced in a time dependent manner over a period of six days of treatment with LMWH (200 µg/ml) in all cell lines (Fig 3.7). The level of TF activity was most rapidly depleted in MDA-MB-231 and SKOV-3 cells, and after six days of treatment with LMWH it was reduced to below 4% of the untreated control. In contrast, the reduction in TF activity in BxPC-3, LoVo and A375 cells was gradual

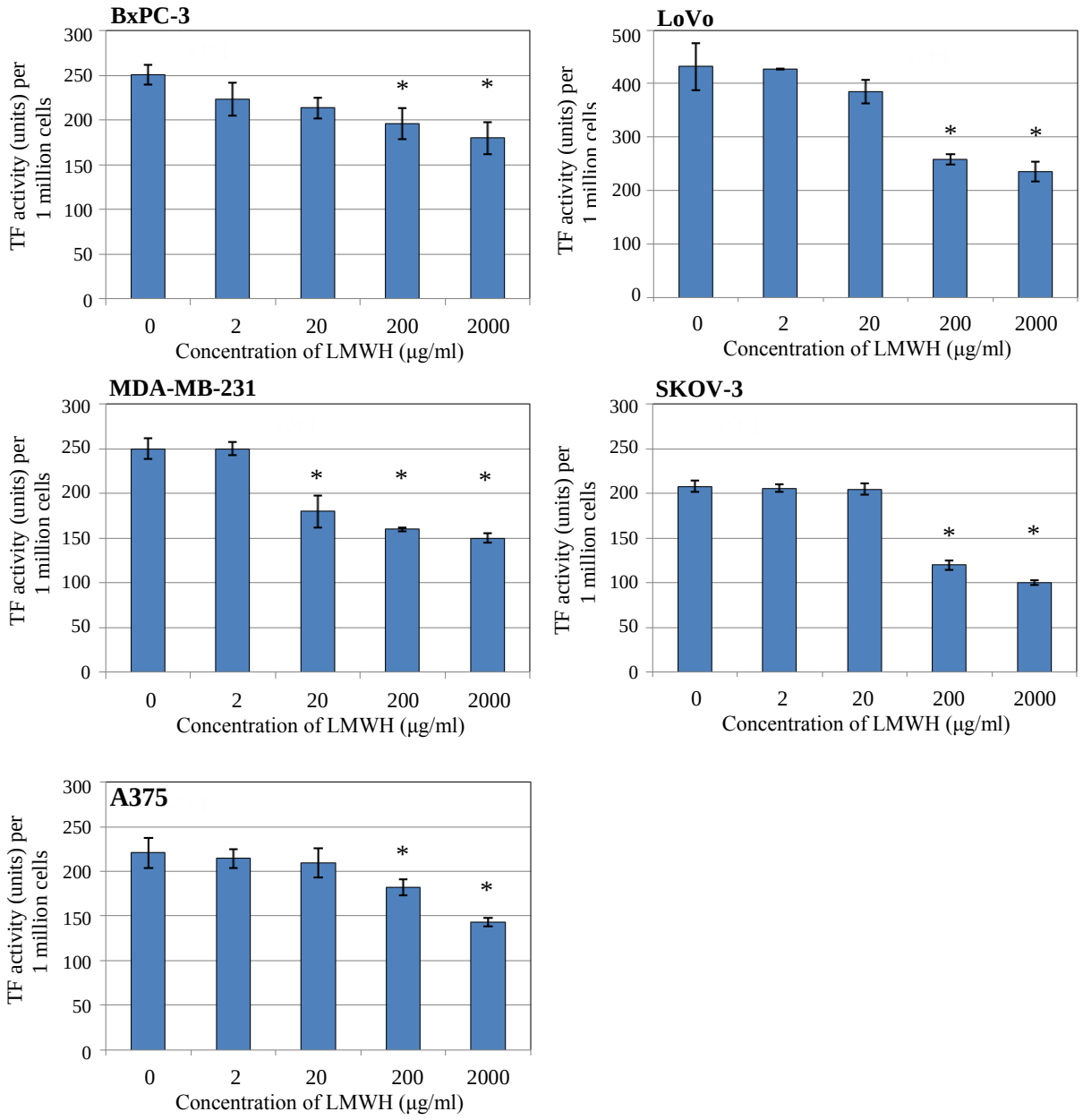

Figure 3. 6 The influence of LMWH on cell surface TF activity

Sets of cells (2×10^5) were incubated with different concentrations of LMWH (0-2000 µg/ml) for 24 h. The cells were resuspended in PBS and cell surface TF activity was measured using a two-stage chromogenic assay. The absorption value of each sample was converted into equivalent concentrations of TF using a standard curve. Data represents the average of three experiments measured in duplicate (*=$p<0.05$ vs. respective untreated sample). Data published in Ettelaie *et al* (2011).

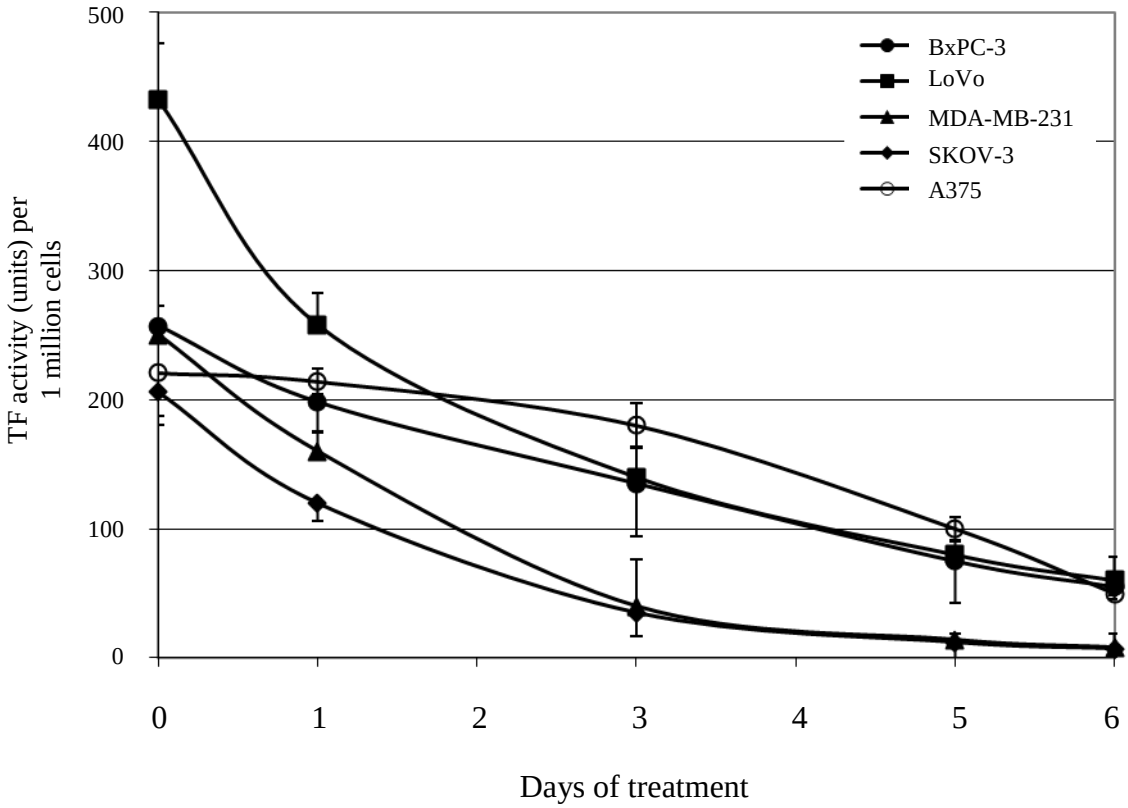

Figure 3. 7 Time-course analysis of the influence of LMWH on the expression of TF activity

Sets of cells (2×10^5) were incubated with LMWH (200 µg/ml) over a period of six days, "0" represents the untreated control samples. The cells were resuspended in PBS and cell surface TF activity was measured using a two-stage chromogenic assay. The absorption value of each sample was converted into equivalent concentrations of TF using a standard curve. Data represents the average of three experiments measured in duplicate (*=$p<0.05$ vs. respective untreated sample). Data published in Ettelaie *et al* (2011).

over the period of six days. However, after six days of treatment with LMWH the level of TF activity was reduced to 21.6%, 13.9% and 22.7% of the untreated control in BxPC-3, LoVo and A375 cells, respectively.

3.4 Influence of LMWH on the transcriptional activity of NFκB

Cells (2×10^5 / well) were transfected with Pathdetect pNFκB-Luc luciferase reporter plasmid using lipofectin and treated with a range of LMWH concentrations (0-2000 µg/ml) for 24 h. The cells were lysed and the luciferase activity of each sample was measured using a luciferase assay. The level of luciferase activity (RLU) corresponds to transcriptional activity of NFκB.

The transfection protocol was previously optimised and the transfection efficiency determined by transfecting the cells with a plasmid capable of expressing GFP (pEGFP-TF) (Ettelaie et al., 2007a, Ettelaie et al., 2007b). Cells (10,000) were then analysed by flow cytometry and a gate was drawn to contain 3% of the untransfected cell population. The transfection efficiency was determined as the percentage of transfected cells within this gate. The transfection efficiency was consistently above 35% in all the cell lines used (Research group of Dr Ettelaie). However, only the luciferase activity of successfully transfected cells was measured so transfection efficiency does not alter the relative amount of cells in which NFκB is activated.

The transcriptional activity of NFκB was reduced at varying concentrations of LMWH in each cell line (Fig 3.8). In BxPC-3 and MDA-MB-231 cells, basal NFκB activity was significantly ($p<0.05$) suppressed in a dose dependant manner following treatment with 20 µg/ml or higher concentrations of LMWH (Fig 3.8). In contrast, suppression of transcriptional NFκB activity was achieved in LoVo, SKOV-3 and A375 cell at higher concentrations of LMWH (200-2000 µg/ml). At the highest concentration of LMWH (2000 µg/ml), transcriptional activity of NFκB was reduced to 28.5% and 46% of the untreated control in BxPC-3 and MDA-MB-231 cells respectively. The transcriptional

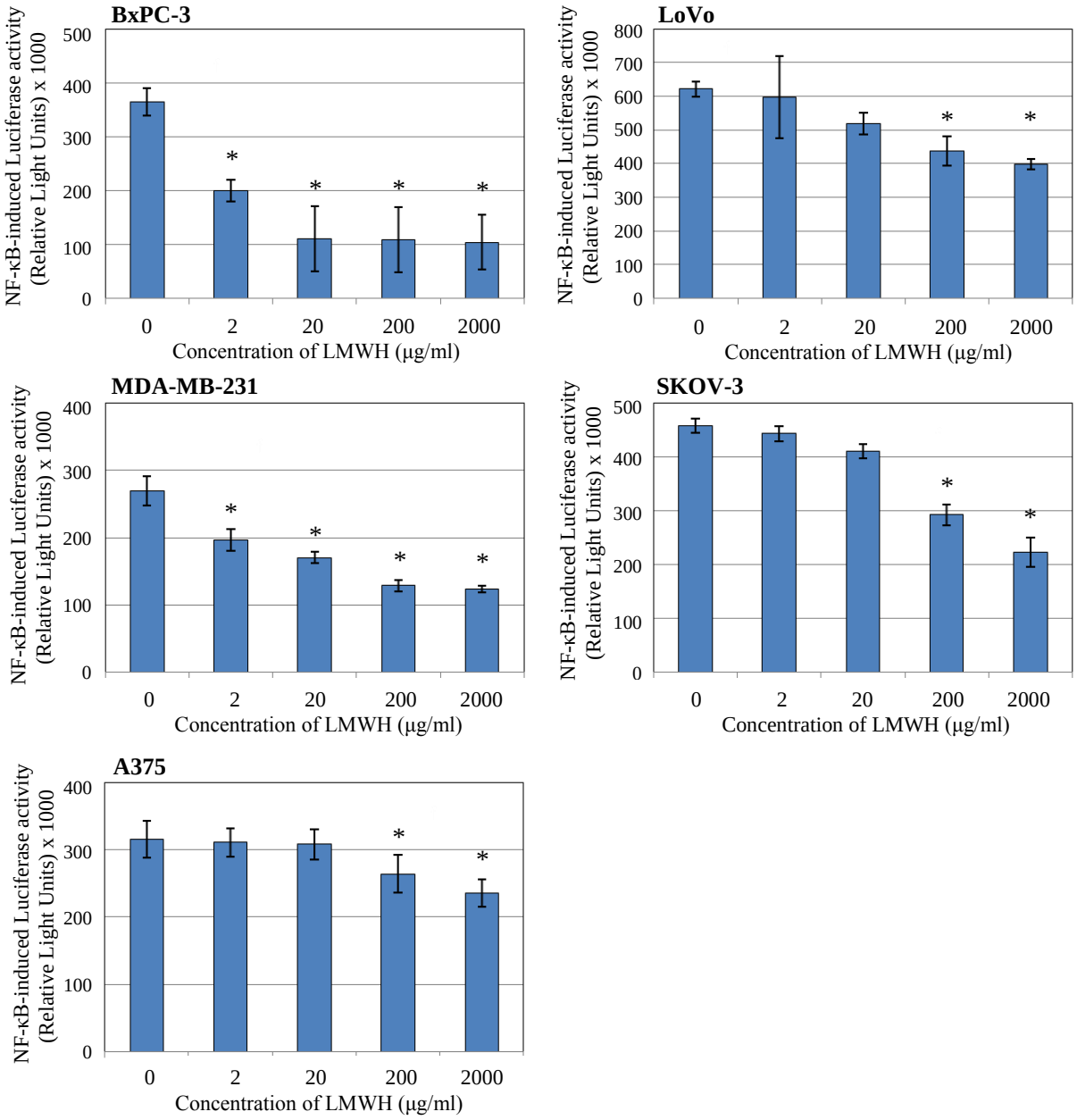

Figure 3. 8 The influence of LMWH on NFκB transcriptional activity

Sets of cells (2×10^5) were transfected with the pNFκB-Luc plasmid. The cells were then incubated with a range of LMWH concentrations (0-2000 µg/ml) for 24 h. The cells were lysed and the luciferase activity in each sample was measured. Data represents the average of three experiments measured in duplicate (*=$p<0.05$ vs. untreated sample). Data published in Ettelaie *et al* (2011).

activity of NFκB was reduced to 48.8% of the untreated control in SKOV-3 cells, but remained above 60% of the untreated control in LoVo and A375 cells.

3.5 The influence of NFκB activity on TF expression

The level of TF antigen was significantly ($p<0.05$) suppressed in all cells following treatment with the NFκB inhibitor pyrrolidinedithiocarbamate ammonium (Fig 3.9). The greatest reduction in detectable total TF antigen, following treatment with pyrrolidinedithiocarbamate ammonium, was observed in MDA-MB-231 and SKOV-3 cells which were reduced to 15.9% and 5.5% respectively of the untreated control. In contrast, the level of total TF antigen did not drop below 60% of the untreated control in BxPC-3 and LoVo cells treated with the NFκB inhibitor. Furthermore, in A375 cells the total TF antigen was not reduced below 80% of the untreated control.

3.6 Influence of supplementation with growth factors on downregulation of TF expression by LMWH.

Cells (2×10^5) were supplemented with a range of concentrations of either EGF (0-10 ng/ml), VEGF (0-4 ng/ml) or bFGF (0-20 ng/ml), in the presence or absence of LMWH (200 µg/ml) and the expression of TF antigen was measured using a TF-specific ELISA. Treatment of cells with EGF (2.5-10 ng/ml) resulted in a dose-dependent increase in expression of TF antigen (Fig 3.10). The greatest increase in expression of TF antigen following treatment with EGF was observed in LoVo, MDA-MB-231, and A375 cells. For example, at the highest concentration of EGF (10 ng/ml) expression of TF antigen was increased by 49%, 112.8% and 58.7% of the untreated control, in LoVo, MDA-MB-231, and A375 cells respectively. Following the inclusion of LMWH (200 µg/ml) to EGF treated cells, the expression of TF antigen was significantly ($p<0.05$) reduced in BxPC-3, MDA-MB-231 and SKOV cells (Fig 3.11). However, higher concentrations of EGF appeared to counteract the influence of LMWH on reducing the expression of TF

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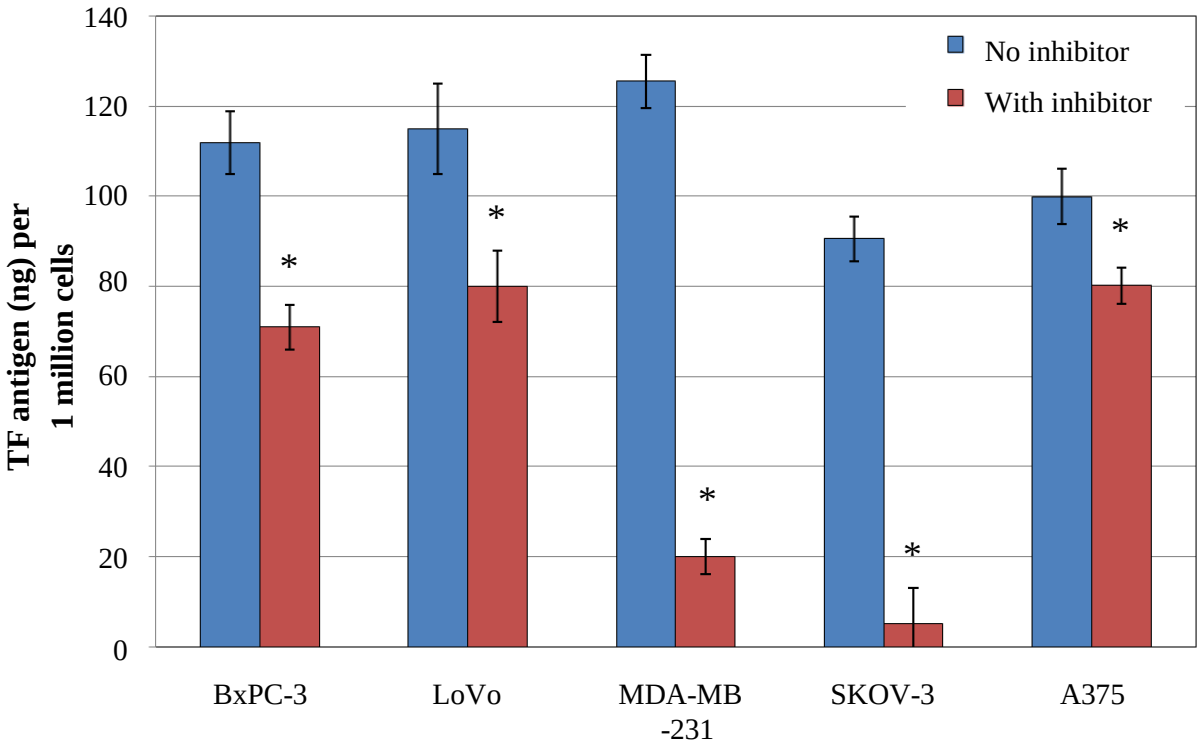

Figure 3. 9 The influence of NFκB inhibitor, pyrrolidinedithiocarbamate ammonium, on TF antigen expression

Sets of cells (2×10^5) were treated for 24 h with NFκB inhibitor pyrrolidinedithiocarbamate ammonium (10 μM) alongside a set of untreated cells and the concentration of TF antigen was measured by ELISA. Data represents the mean of three experiments measured in duplicate (*=$p<0.05$ vs. respective untreated sample). Data published in Ettelaie *et al* (2011).

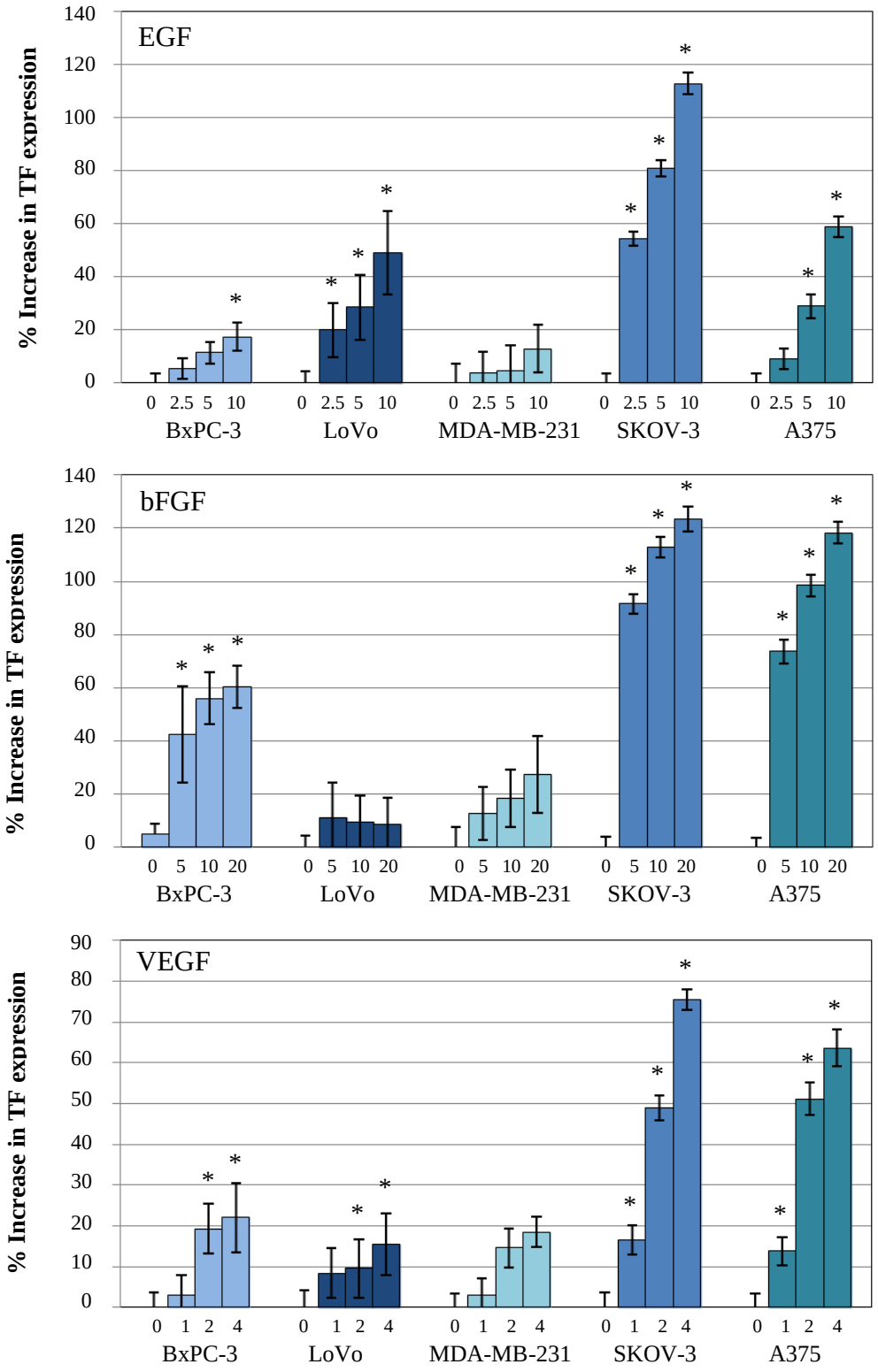

Figure 3. 10 The influence of EGF, bFGF and VEGF on TF expression

Sets of cells (2×10^5) were supplemented with a range of concentrations of EGF (0-10 ng/ml), bFGF (0-20 ng/ml), or VEGF (0-4 ng/ml) for 24 h. The cells were lysed and analysed by ELISA, TF concentration was determined from a standard curve prepared alongside. The percentage increase in TF antigen was determined against untreated samples. Data represent the average of three experiments measured in duplicate (*=$p<0.05$ vs. untreated sample). Data published in Ettelaie *et al* (2011b).

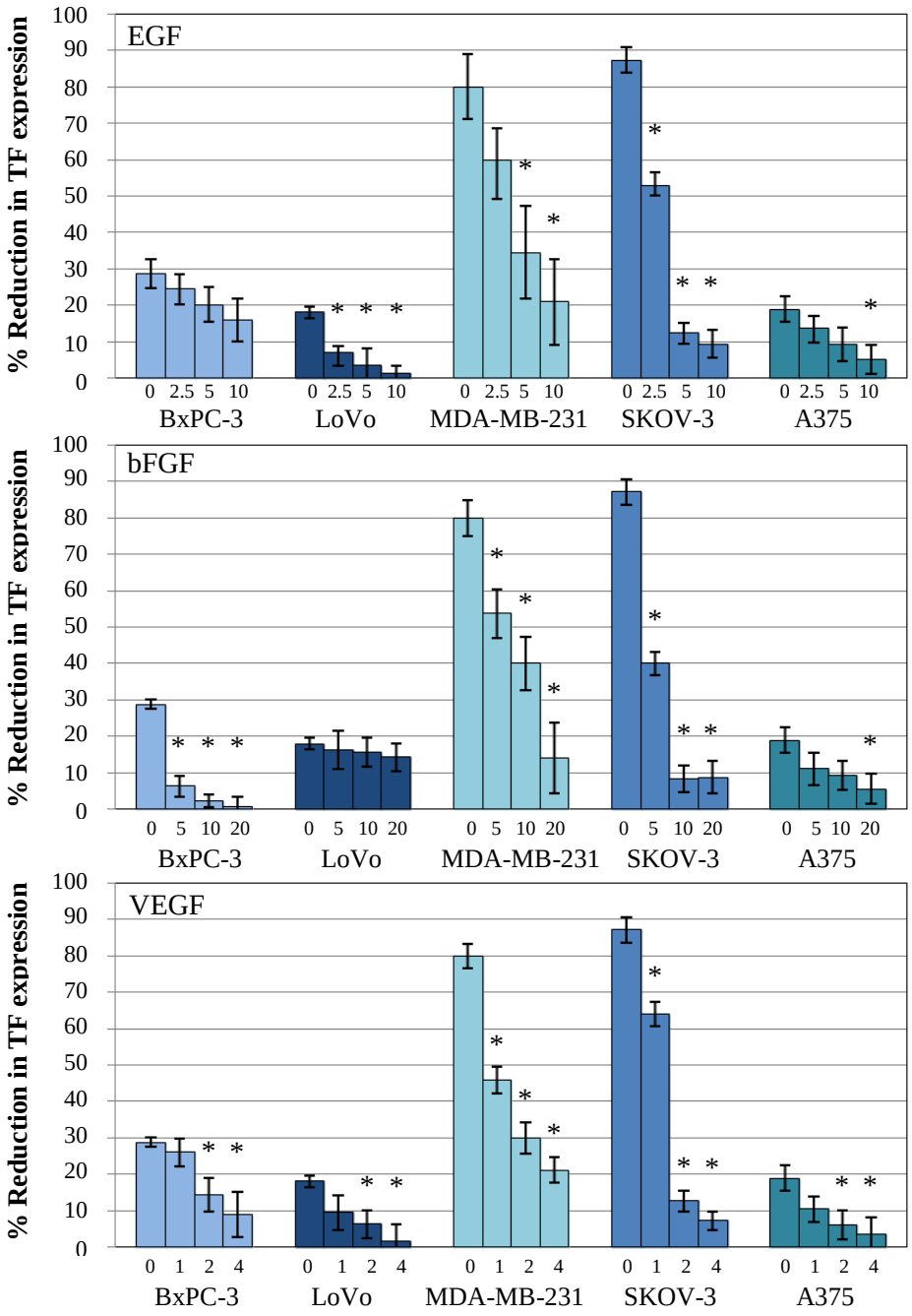

Figure 3. 11 The influence of EGF, bFGF and VEGF on the downregulation of TF expression by LMWH

Sets of cells (2×10^5) were supplemented with a range of concentrations of EGF (0-10 ng/ml), bFGF (0-20 ng/ml), or VEGF (0-4 ng/ml) in the presence of LMWH (200 µg/ml) for 24 h. The cells were lysed and analysed by ELISA, TF concentration was determined from a standard curve prepared alongside. The percentage reduction in TF antigen was determined against samples treated with the same concentration of growth factor but without treatment with LMWH. Data represent the average of three experiments measured in duplicate (*=$p<0.05$ for the alteration in the level of TF suppression against the respective sample, without growth factor). Data published in Ettelaie *et al* (2011b).

antigen in Bx-PC-3, MDA-MB-231, A375 cells and SKOV-3 cell.

Incubation of cells with bFGF (5-20 ng/ml) resulted in a dose-dependent increase in expression of TF antigen (Fig 3.10). The greatest increase in TF antigen expression were observed in BxPC-3, SKOV-3 and A375 cells, which were increased by 60.3%, 123.4% and 118.3% respectively, of the untreated control, at the highest concentration of bFGF (20 ng/ml). Furthermore, the inclusion of LMWH (200 μg/ml) to bFGF treated cells, reduced the expression of TF antigen in all cells (Fig 3.11). However, the influence of LMWH on TF expression was counteracted at higher concentrations of bFGF, which was most evident in BxPC-3, MDA-MB-231 and SKOV-3 cells.

Finally, supplementation of cells with VEGF (1-4 ng/ml) resulted in a dose-dependent increased expression of TF antigen, with the greatest increases observed in SKOV-3 and A375 cell which were increased by 75.5% and 63.7% respectively, of the untreated control, at the highest concentration of VEGF (Fig 3.10). Although the inclusion of LMWH (200 μg/ml) to VEGF treated cells reduced the expression of TF antigen in all cells, higher concentrations of VEGF counteracted the reduction (Fig 3.11). This counteraction was most evident in MDA-MB-231 and SKOV-3 cells.

3.6 Influence of LMWH on cell invasion

A Boyden chamber based invasion assay was used to examine the influence of LMWH on cell invasion. The ability of cells to cross the collagen type IV barrier and invade the lower Boyden chamber was reduced with the inclusion of LMWH (Fig 3.12). The number of BxPC-3 and MDA-MB-231 cells across the collagen coated barrier was significantly ($p<0.05$) reduced compared to untreated controls, when treated with concentrations of LMWH 20 μg/ml, or above. At the highest concentration of LMWH (2000 μg/ml) cell invasion was reduced to 25.4% and 41.6% of the untreated control, in BxPC-3 and MDA-MB-231 cells respectively. In contrast, the lowest concentration of

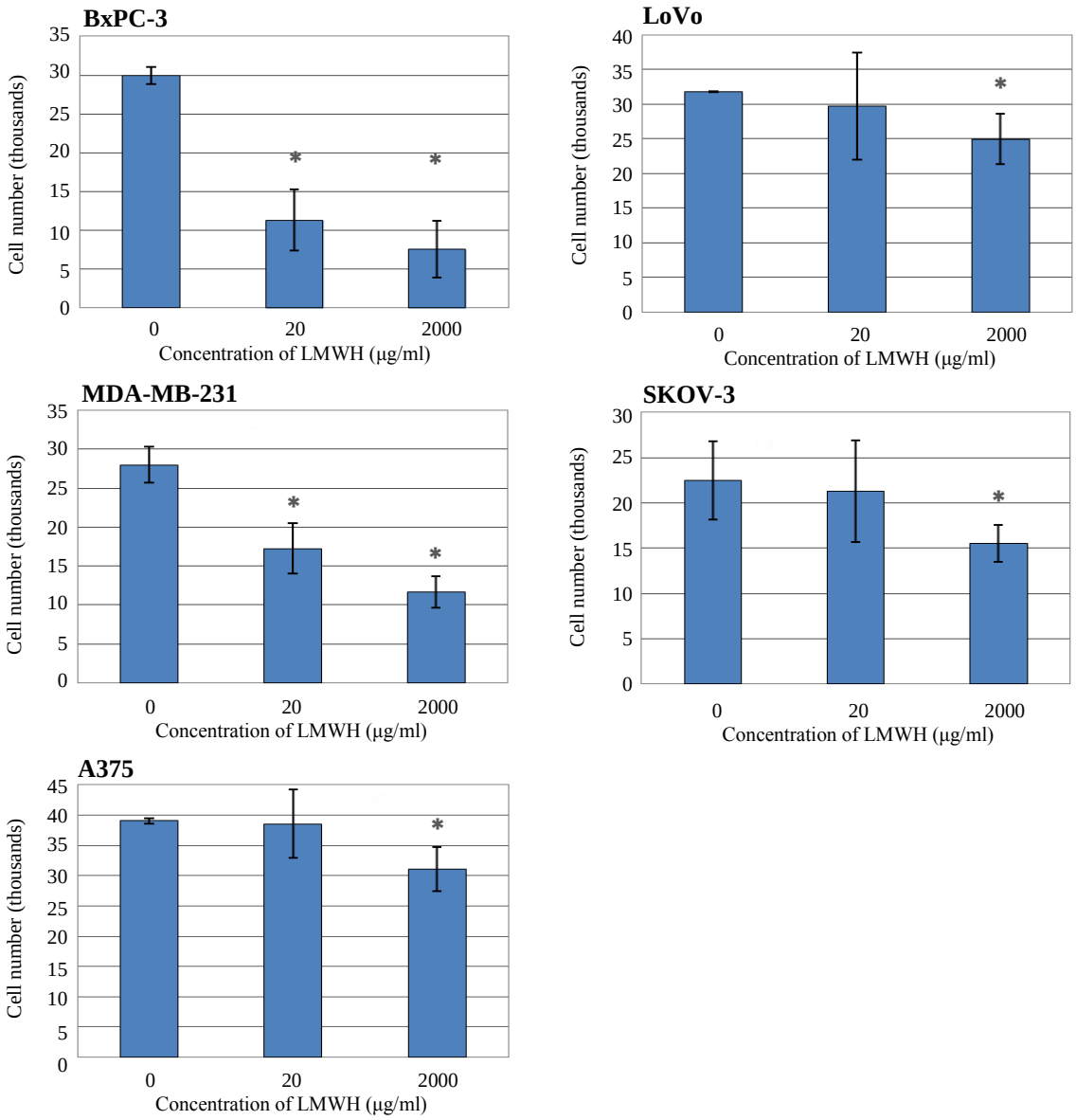

Figure 3. 12 The influence of LMWH on cell invasion

Cells (5×10^4) were seeded in the upper compartment of Boyden chambers and treated with a range of LMWH concentrations (0, 20, and 2000µg/ml). The cells were incubated for 24 h at 37°C to allow the cells to cross the collagen barrier. The migrated cells were fixed with glutaraldehyde, stained with crystal violet and 1 % SDS was added to solubilise the stained cells so that the crystal violet was released by the cells into the SDS solution. The absorption of each sample was measured at 595 nm on a spectrophotometer and the number of cells that migrated across the collagen barrier was determined from a standard curve prepared alongside. Data represents the average of three experiments measured in triplicate ± SD. (*$p<0.05$ vs. untreated sample). Data published in Ettelaie *et al* (2011a).

LMWH (20 μg/ml) had no significant ($p<0.05$) effect on the invasion rate of LoVo, SKOV-3 and A375 cells. Furthermore, although the highest concentration of LMWH (2000 μg/ml) significantly ($p<0.05$) reduced the rate of invasion of LoVo, SKOV-3 and A375 cells, the number of cells to cross the collagen coated barrier was not reduced to below ~ 70% of the untreated control.

CHAPTER 4

Discussion

4.0 Discussion

Various studies have reported the benefits of treating cancer patient with LMWH, in relation to increased survival rates (Kakkar et al., 2004, von Delius et al., 2007). Moreover, studies have suggested that the benefits of treating cancer patients with anticoagulants, such as LMWH, are not due to direct anti-tumour effects, instead they have been attributed to the suppression of circulating TF, which is increased in many types of cancer and is associated with more aggressive disease, and in thrombosis (Signaevsky et al., 2008). In this study the influence of LMWH on suppression of cellular TF expression and activity was examined. The underlying mechanisms by which LMWH suppresses TF expression in cancer cells were also investigated. In addition, the effect of suppression of TF expression by LMWH on cancer cell invasiveness was investigated. Prior to this study all five cell lines used here had been confirmed to express significantly high levels of TF mRNA, antigen and cell surface activity (data presented as untreated control samples), and were therefore considered appropriate for use in this study (Research group of Dr Ettelaie). Prior to this study a trypan blue exclusion assay was used to confirm that there was no significant difference in cell viability between LMWH treated cells and the untreated control cells (Research group of Dr Ettelaie).

Initially, the influence of LMWH on the expression of TF mRNA, antigen and activity in cancer cell lines was investigated. A trend was observed in which incubation of all cell lines with high concentrations of LMWH (200-2000 μg/ml) resulted in significant suppression of TF mRNA expression (Fig 3.2). Despite LoVo and MDA-MB-231 cells exhibiting the highest baseline expression of TF mRNA they were the most sensitive to treatment with LMWH, exhibiting the greatest reductions in expression of TF mRNA. The suppression of TF mRNA in all cells coincided with a reduction in the level of

detectable total TF antigen, however not to the same extent. Although the highest concentrations of LMWH (200-2000 μg/ml) did significantly reduce the level of TF antigen in all cell lines, the decrease in TF antigen did not fall below 60%, of the untreated control, in BxPC-3, LoVo or A375 cells (Fig 3.4). In addition, the reduction of TF antigen in BxPC-3, LoVo and A375 cells, correlated with a moderate decrease in TF activity, which did not drop below 55% of the untreated control (Fig 3.6). As TF antigen and activity were not immediately depleted on suppression of TF mRNA by LMWH, it is possible that the presence of intracellular reservoirs of TF are responsible for replenishing cell surface TF even after treatment with LMWH. This would help explain why the level of TF antigen remained high in LoVo cells despite the rapid downregulation of TF mRNA expression. Furthermore, the treatment of BxPC-3, LoVo and A375 cells with LMWH over a period of six days resulted in the gradual depletion of TF antigen (Fig 3.5), and activity (Fig 3.7). This again supports the presence of intracellular reservoirs of TF which are gradually depleted as they replenish cell surface TF. In support of this hypothesis, various studies have demonstrated that lysed cells have higher TF activity than TF measured with intact cells (Bona et al., 1987, Carson and Archer, 1986, Carson et al., 1990, Maynard et al., 1977). This has led to the development of the concept that TF exists in various intracellular pools within cells (Nemerson and Giesen, 1998, Schecter et al., 1997). Studies in a variety of cell types have found that a substantial proportion (up to 90%) of TF antigen and activity exists in a latent form on or near the cell surface (Bach and Moldow, 1997, Carson, 1996, Drake et al., 1989, Maynard et al., 1975, Rao et al., 1992). However, more recent studies have also demonstrated that a substantial amount of TF is located intracellularly, but have suggested that the TF generally accumulates in a distinct perinuclear pattern (Camera et al., 1999, Egorina et al., 2005, Fortin et al., 2005, Hansen et al., 2001, Schecter et al., 1997). Attempts to breakdown the proportion of total TF activity in latent and

intracellular pool, in stimulated smooth muscle cells, estimated that ~20% of cellular TF is available on the cell surface, ~30% was active intracellular TF and the remaining TF was latent (Schecter et al., 1997). In contrast, the localisation of TF in fibroblasts suggests that 75% of total TF antigen is localised intracellularly and is not functionally active (Mandal et al., 2006). As the localisation of TF in latent and intracellular pools appears to be cell type specific, further investigation using immunostaining would be required to confirm the presence and localisation of intracellular pools of TF in BxPC-3, LoVo and A375 cells.

In contrast with the above, the treatment of MDA-MB-231 and SKOV-3 cells with LMWH for 24 h resulted in significant reductions in the level of TF antigen (Fig 3.4), and to a lesser extent TF activity (Fig 3.6). Moreover, the level of TF antigen and activity remained consistently low over a period of six days of treatment with LMWH (Fig 3.4 & Fig 3.6). Therefore it possible to hypothesise that the majority of cellular TF is present on the surface of MDA-MB-231 and A375 cells, which is rapidly transferred and released into the media following treatment with LMWH. The rapid release of TF in response to LMWH helps to explain the rapid reduction of TF antigen in SKOV-3 cells, despite a moderate suppression in the level of TF mRNA expression. In support of this hypothesis, studies have reported the rapid release of TF by arterial smooth muscle cells under flow conditions, which demonstrates that cells can rapidly release TF when exposed to a different environment (Stampfuss et al., 2006).

The release of TF-bearing microparticles are thought to be an important link between cancer and thrombosis (Tesselaar et al., 2007), with numerous studies reporting high levels of TF-bearing microparticles in cancer patients (Del Conde et al., 2007, Tesselaar et al., 2007). During the present study, the level of TF microparticle release was not investigated. Subsequent studies have demonstrated that despite the observed reduction in TF antigen and cell surface TF activity, the concentration of released TF activity is

not altered upon incubation with increasing concentration of LMWH (0-2000 μg/ml) in any of the cell lines (Research group of Dr Ettelaie) (Ettelaie et al., 2011b). However, the prolonged treatment of all cells with LMWH (200 μg/ml) resulted in a progressive decrease in microparticle- derived TF (Ettelaie et al., 2011b). The data further supports the presence of intracellular reservoirs of TF, which are gradually reduced over prolonged periods of time as they replenish the surface TF.

The LMWH used throughout the study was obtained from Sigma Chemical Company Ltd, and confirmed using the commercially available dalteparin (FRAGMIN®). The study has to take into account that different LMWH's differ in molecular composition and pharmacological properties, depending on their method of preparation (Barrowcliffe, 1995, Fareed et al., 1998). As a result, different LMWH's used in the prophylactic treatment of cancer patients may have varying anti-neoplastic properties. The concentrations of LMWH used throughout this investigation, 20-2000 μg/ml (activity 0.1-10 unit/ml), are comparable to therapeutic levels of LMWH reported to be used in the prophylactic treatment of cancer patients (Gerotziafas and Samama, 2004, Zanon et al., 2005). Therefore, it is likely that suppression of TF expression in cancer patients is realistic at these therapeutic doses of LMWH.

The gradual depletion of TF expression and activity over time suggests that cancer patients should be treated with LMWH over a prolonged period of time in order to be effective at treating the TF-induced hypercoagulable state. In support of this, prolonged treatment with LMWH has previously been reported as being beneficial to cancer patients with less advanced disease by increasing survival time (Kakkar et al., 2004). Furthermore, the results from this study are consistent with previous *in vivo* studies which found that prophylactic treatment of advanced pancreatic cancer patients with dalteparin may result in a decrease in circulating TF antigen levels compared to the placebo treated group (Maraveyas et al., 2010). Reductions in plasma TF levels

following administration of heparin have also been observed in patients with acute myocardial infarction (Yamamoto et al., 2000), and in patients with angina pectoris (Soejima et al., 1999). In patients with acute myocardial infarction the level of plasma TF was maintained at a consistently low level during continuous heparin infusion, but increased when heparin administration ceased (Yamamoto et al., 2000). In conflict with the data from this study, a previous study using a mouse xenograft model of cancer found LMWH had no significant effect on procoagulant TF activity (Niers et al., 2009). However, the mouse model only used a single bolus injection which was administered prior to the injection of cancer cells. In contrast, the data from this study indicates that prolonged treatment with LMWH (200 μg/ml), is most affective at suppressing TF antigen expression (Fig 3.5), and TF activity (Fig 3.7), with a progressive decrease being observed over a period of six days.

Next, the influence of LMWH on NFκB transcriptional activity was investigated. The transcriptional activity of NFκB was suppressed at approximately the same effective concentrations of LMWH that TF mRNA was downregulated in each cell line. This was with the exception of BxPC-3 and MDA-MB-231 cells, in which NFκB transcriptional activity was reduced to a greater extent than TF mRNA at lower concentrations of LMWH (Fig 3.8). However, although LMWH suppressed NFκB activity in all cell lines the suppression was not attenuated proportionally to the suppression of TF mRNA. It is known that NFκB is responsible for the transcription of TF as the promoter region of the TF gene contains an NFκB binding site (Mackman, 1997). However, in addition to the NFκB binding site, the TF promoter region also contains binding sites for AP1, SP1 and EGR1 through which TF mRNA may also be transcribed (Fig 4.1) (Mackman, 1997, Mackman, 2001). Therefore although transcriptional activation of NFκB may contribute to a large proportion of the TF expressed by these cells, the contribution of other transcription factors to the regulation of TF expression cannot be ruled out. This may

explain why NFκB activity was reduced to a greater extent than TF mRNA expression in BxPC-3 and MDA-MB-231 following treatment with LMWH. It is also possible that LMWH may be interfering with the other transcription factors that are known to regulate TF expression, however further investigation would be needed to confirm this.

The specificity of NFκB as the regulator of TF expression in the cells used in this study, was confirmed using the NFκB inhibitor, pyrrolidinedithiocarbonate ammonium. The inhibition of NFκB resulted in a decrease in the level of TF antigen which was comparable to the reduction achieved with LMWH (Fig 3.9). As with LMWH treatment, the level of TF antigen remained above 60% of the untreated control in BxPC-3, MDA-MB-231 and A375 cells following inhibition of NFκB. Therefore, consistent with the results above, the level of TF antigen may not have been immediately depleted, upon inhibition of NFκB, because these cells may contain TF within intracellular reservoirs. In addition, it is also possible that other transcription factors may be contributing to the expression of TF, which may also help to explain why the level of TF antigen was not completely diminished upon inhibition of NFκB. Subsequent experiments have demonstrated that betulinic acid, used to activate NFκB, reverses the suppression of TF antigen by LMWH, which further supports the involvement of NFκB in the mechanism of inhibition by LMWH (Research group of Dr Ettelaie) (Ettelaie et al., 2011b).

The engagement of growth factor receptors is known to activate transcriptional activity of NFκB (Graham and Gibson, 2005, Moulik et al., 2008, Ulbrich et al., 2008). Studies have reported NFκB signalling downstream of a number of growth factor receptors, including platelet-derived growth factor, epidermal growth factor receptor and nerve growth factor receptor (Habib et al., 2001, Maggirwar et al., 1998, Romashkova and Makarov, 1999). Furthermore, the increased expression of growth factor receptors has

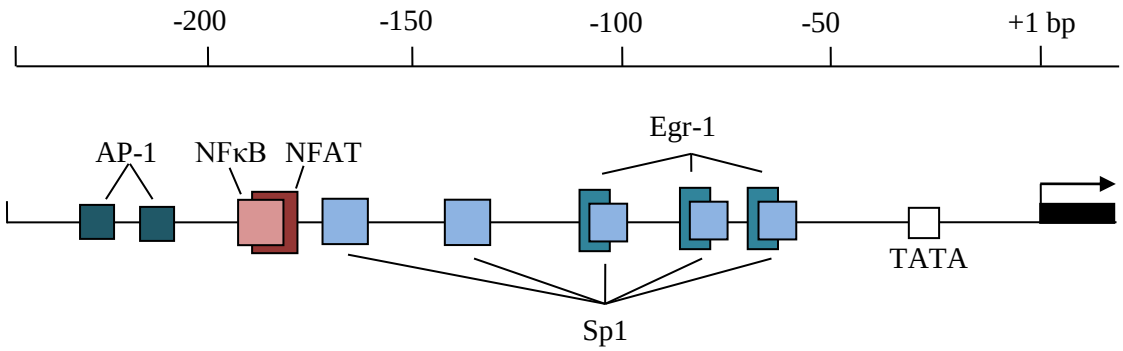

Figure 4. 1 Human TF promoter

The TF promoter contains binding sites for various transcription factors including NFκB, AP1, SP1 and EGR1, which regulate TF gene expression. The arrow indicates the start of transcription. Adapted from Mackman (2001).

been implicated as being one of the underlying causes of the hyperactivity of cancer cells to growth factors (Bucci et al., 1997, Moehler et al., 2008). Numerous studies have reported that overexpression of growth factor receptors can be correlated with increased tumour invasion, metastasis and the poor prognosis of cancer patients (Hu et al., 2007, Onogawa et al., 2004, Sebastian et al., 2006, White et al., 2002). Therefore, this study hypothesised that LMWH suppresses NFκB, and subsequently TF expression by interfering with growth factor receptor signalling. The effectiveness of LMWH in suppressing expression of TF antigen was measured in the presence of increasing concentrations of EGF, bFGF, and VEGF. The interaction of heparin with growth factors, and their respective growth factor receptors has previously been reported. For example, studies have reported a decrease in the level of bFGF binding to cell surface receptors when exposed to high concentrations of heparin (Fannon et al., 2000). Likewise, studies have also demonstrated that increasing concentrations of heparin reduce the affinity of VEGF for their surface receptors (Ito and Claesson-Welsh, 1999). However, the interaction of EGF with heparin has not been reported. Each cell line used in this study exhibited distinct sensitivities to the different growth factors, meaning increasing concentrations of growth factors increased the expression of TF antigen to different extents (Fig 3.10). It is possible to suggest that each cell line expresses different growth factor receptors on their surface, however this would need to be confirmed by immunohistochemisty staining. In addition, it is possible that the engagement of the different growth factors with their respective cell surface receptors may not initiate downstream pathways which regulate the expression of TF. In support of this theory one study suggested that despite the association of EGF with increased breast cancer risk, EGF does not regulate TF expression (Kato et al., 2005). This could explain why TF antigen expression was not significantly increased in MDA-MB-231 cells supplemented with EGF. Likewise, this could also help explain why the expression

of TF antigen is not increased in LoVo cells treated with bFGF, but is significantly increased in SKOV-3 cells treated with all concentrations of bFGF.

The increase in expression of TF was reduced in growth factor supplemented cells following the inclusion of LMWH. However, since each cell line displayed distinct sensitivities to certain growth factors the expression of TF antigen was suppressed by LMWH to different extents (Fig 3.11). For example, SKOV-3 cells were highly sensitive to treatment with all growth factors, which meant that at high growth factor concentrations the expression of TF was reduced by LMWH to a lesser extent, than previously observed (Fig 3.4). Additionally, lower concentrations of LMWH were not effective at reducing TF expression in LoVo and A375 cells (Fig 3.4), which was reflected in the inability of LMWH to reduce TF expression in the presence of growth factors (Fig 3.11). Studies have reported functional mutations in growth factor receptors and their associated proteins on tumour cells (Hynes and Lane, 2005, Stephens et al., 2004), therefore it is possible to speculate that LoVo and A375 may have functional mutations, which could mean TF expression is not under normal cellular regulation and explain why LMWH is less affective at reducing TF expression.

In general this data supports the hypothesis that LMWH may interfere with the interactions of the growth factors and their respective cell surface receptors. However, the exact mechanism by which LMWH does this has not been elucidated in this study. One possible scenario is that LMWH binds to the growth factors preventing them from binding with their growth factor receptors. This would help explain why the ability of LMWH to reduce TF expression was reduced at high concentrations of growth factors, as it is possible LMWH became saturated at high concentrations of growth factors so the excess growth factors were able to efficiently bind with their respective growth factor receptors and initiate downstream expression of TF. In support of this, various other studies have suggested that many growth factors bind to heparin with high affinity

(Conrad, 1998, Fannon et al., 2000, Powell et al., 2004). In addition, although most growth factors activate downstream signaling by binding with cell surface growth factor receptors, various studies have demonstrated that downstream signaling can also be mediated through growth factors binding with high affinity to heparan sulphate proteoglycans on the surface of cells (Fannon et al., 2000, Forsten-Williams et al., 2008). Therefore, as growth factors are known to bind with high affinity to both heparin and heparan sulphate on the surface of cells, the binding of growth factors with LMWH instead of cell surface heparan sulphate may be contributing to the reduced downstream expression of TF observed in this study. However, further investigation would be required to confirm this mechanism.

In addition to its role in the prothrombotic state of cancer patients, TF is also thought to play an important role in cancer cell progression and metastasis (Kato et al., 2005, Ohta et al., 2002, Wan et al., 2002). High TF expression is associated with increased invasive and metastatic potential in many types of malignancies (Kato et al., 2005, Konigsberg et al., 2001). As described in above, LMWH is capable of suppressing TF expression, so the ability of LMWH to reduce cell invasiveness was examined. The ability of tumour cells to degrade and traverse epithelial and endothelial basement membranes plays a crucial role during metastasis (Liotta et al., 1980). One of the major components of the basement membrane is type IV collagen (LeBleu et al., 2007). Therefore, the ability of cancer cell lines to degrade a collagen-IV membrane and migrate towards a stimulus, in the presence of LMWH, was measured using a Boyden chamber based assay. Treatment of cells with LMWH (0-2000 μg/ml) resulted in a dose dependant decrease in cell invasion across the collagen-IV membrane. Inhibition of cell invasion was most significant in BxPC-3 and MDA-MB-231 cells (Fig 3.12). Subsequent studies have since found that overexpression of TF in BxPC-3 and MDA-MB-231 cells restored cell invasiveness, even after treatment with LMWH (Research group of Dr Ettelaie).

(Ettelaie et al., 2011a). The reduction in cell invasiveness correlates with the observed reduction in NFκB activity, expression of TF antigen and TF activity following treatment with LMWH. Therefore, the data suggests that LMWH may be reducing cancer cell invasion through mechanisms partially attributed to the suppression of TF expression and activity through the downregulation of NFκB. Subsequent studies have shown that the observed reduction in TF antigen and activity, following treatment with LMWH, strongly correlated with significant reductions in cancer cell migration (Research group of Dr Ettelaie) (Ettelaie et al., 2011a). In support of this data, studies have demonstrated that cell migration is enhanced by TF-FVIIa-mediated signaling, through PAR2 activation, in human breast cancer cells known to express high levels of cell surface TF (Jiang et al., 2004). This could explain why cell invasiveness remained high in LoVo and A375 cells, because the level of cell surface TF activity remained highest in these cell lines following incubation with LMWH (200 μg/ml) for 24 h (Fig 3.7).

Studies have demonstrated that TF can increase the invasive ability of human colon cancer cells through mechanisms thought to involve the regulation of metalloprotease expression (Tang et al., 2005, Zhang et al., 2008). The matrix metalloprotease family consists of numerous Zn – dependent endopeptidases which are collectively capable of degrading all components of the extracellular matrix (Birkedal-Hansen et al., 1993, Sternlicht and Werb, 2001). Moreover, matrix metalloproteases are thought to play a role in the creation of the proteolytic defects in basement membrane type IV collagen that allow tumour cell invasion (Ray and Stetler-Stevenson, 1994, Nabeshima et al., 2002). Therefore, it is possible that by suppressing TF expression and activity, LMWH may be indirectly reducing cancer cell invasion by reducing the regulation of metalloproteases. In addition, it is well established that TF induces chemotaxis, the process by which cells direct their movements according to environmental conditions

(Gessler et al., 2010, Siegbahn et al., 2005). For example, one study demonstrated that the phosphorylation of cytoplasmic domain of TF is necessary to potentiate the chemotactic response of cells to platelet derived growth factor (Siegbahn et al., 2005). Therefore, it is possible that by suppressing the level of TF antigen and activity, LMWH may be reducing cell mobility, which in turn could be beneficial in lowering the rates of metastasis in cancer patients. Further investigation would be required to confirm either of these hypotheses.

4.1 Conclusion

Various studies have previously reported the beneficial effect of LMWH in the treatment of cancer patients, which many have attributed to the suppression of circulating TF (Gori et al., 1999, Kakkar and Williamson, 1997). The data from this study indicates that LMWH is capable of downregulating both TF expression and activity in the cancer cell lines used during this study, in a dose and time dependent manner. In BxPC-3, LoVo and A375 cells, the depletion of TF antigen and activity was gradual over a prolonged period of time, which could indicate the presence of intracellular reservoirs of TF which replenish TF on the cell surface. Therefore, the gradual depletion of intracellular reservoirs, achieved by prolonged treatment with LMWH, could be beneficial in reducing the level of procoagulant cellular TF, and the amount of TF-bearing microparticles released by tumour cells. In part, the mechanisms by which LMWH downregulates TF expression appears to involve the suppression of transcriptional activity of NFκB. However, the data suggests that transcription factors other than NFκB may be involved in the regulation of TF. It appears that LMWH may be capable of reducing the transcriptional activity of NFκB by interfering with the interactions of growth factors and their respective cell surface receptors. Furthermore, the data indicates that LMWH suppresses cellular invasion in all cell lines, through

mechanisms which appear to correlate with the downregulation of NFκB activity and the subsequent suppression of TF expression and activity. In conclusion, it is possible to suggest that the benefits of LMWH therapy may extend beyond the immediate inhibition of coagulation and that long term therapy could be advantageous in limiting the expression of TF, which in turn could be beneficial in reducing the rate of tumour cell invasion.

4.2 Further Investigation

During the course of this investigation there has been increasing amounts of evidence to suggest the presence of intracellular reservoirs of TF. Various studies have used immunostaining to characterise the localisation of TF (Schecter et al., 1997). Therefore, further investigation could involve the immunostaining of TF in each cell line, which would confirm the existence and localisation of intracellular reservoirs of TF. This method could also be employed to analyse the effect of LMWH on the distribution of TF in each cell line.

As previously mentioned, other transcription factors, such as AP1, SP1 and EGR1, are known to be involved in the regulation of TF (Mackman, 1997). Therefore further investigation could be carried out to analyse the influence of LMWH on these transcription factors. This could be carried out using a luciferase reporter system, as previously described for analysis of transcriptional activity of NFκB.

It is worth noting that there was some variation in the quality of Bradford assay standards between each set of ELISA experiments (Fig 2.1). This may have meant that the amount of protein loaded during each ELISA was not consistently accurate, which could have caused inaccuracies in the actual amount of TF antigen detected in each sample. The data represents an average of 4 experiments in duplicate and the data for all cell lines had relatively low standard deviation, so the data should be considered an

accurate representation of amount of TF antigen expressed by each cell line. However, in any further investigation an alternative to using ELISA could be to use western blotting. This method would allow the amount of TF protein in each sample to be quantified using densitometry, and the amount of protein loaded for each sample could be checked using a β-actin antibody.

Mitogen-activated protein kinases (MAPKs), such as Jun N-terminus kinase (JNK), P38 and Erk have been shown to play a crucial role in cell migration (Jiang et al., 2004, Huang et al., 2004). MAPK pathways are known to be induced by a wide variety of stimuli including hormones, growth factors and receptor tyrosine kinases (Kyriakis and Avruch, 2001, Katz et al., 2007). Moreover, various studies have documented the involvement of MAPKs in the regulation of TF expression (Yu et al., 2005, Vega-Ostertag et al., 2005, Rong et al., 2009, Eto et al., 2002). Interestingly, recent studies have demonstrated that treatment of melanoma cells with LMWH inhibited cell migration through mechanisms thought to involve a reduction in activation of JNK (Chalkiadaki et al., 2011). It is possible to speculate that by interfering with the interactions of growth factors with their cell surface receptors, LMWH may be reducing the activation of MAPKs, which in turn could contribute to reducing the regulation of TF expression. Therefore, further investigation into the possible mechanisms involved in the downregulation of TF expression by LMWH could involve examining the effect of LMWH on regulation of MAPKs.

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