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Studies on Tissue Factor with Focus on Cell Signaling and Cancer

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Abstract

Eriksson, O. 2015. Studies on Tissue Factor with Focus on Cell Signaling and Cancer. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1075. 63 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9180-2.

This thesis have explored the functions of the protein Tissue Factor (TF), which together with its ligand coagulation factor VII/VIIa (FVII/FVIIa) forms a proteolytic complex that functions in initiation of blood coagulation and activation of cell signaling.

In paper I, the mechanisms behind the observation that TF/FVIIa signaling protects cells from apoptosis were further investigated. Using cell culture models, we found that antiapoptotic signaling by TF/FVIIa requires signaling by the Insulin-like growth factor I receptor (IGF-1R), as synthetic IGF-1R inhibitors and IGF1-R siRNA knock-down abolished the antiapoptotic effect of FVIIa. Furthermore, the IGF-1R translocated to the cell nucleus after FVIIa stimulation, implying a role in regulation of gene expression.

Papers II and III describe the discovery that the Eph tyrosine kinase receptors EphB2 and EphA2 are proteolytically cleaved directly by TF/FVIIa. By using mass spectrometry and N-terminal Edman sequencing, the exact cleavage site was identified after a conserved arginine residue in the EphA2/EphB2 ligand binding domains, in agreement with the cleavage preferences of FVIIa. TF and EphA2/EphB2 co-localized in cancer cell lines and FVIIa potentiated ligand-dependent Eph signaling by increasing cytoskeletal remodeling and cell repulsion, demonstrating a novel proteolytical event that modulates Eph receptor signaling.

In paper IV, expression of TF was investigated in colorectal cancer in both the stromal and tumor cell compartments by immunohistochemistry using an anti-TF-antibody developed and validated by the Human Protein Atlas project. In normal large intestine, TF was strongly expressed in the innermost pericryptal sheath cell layer lining the epithelium, in a cell population distinct from intestinal pericryptal myofibroblasts. We evaluated TF expression in two colorectal cancer materials, and found that TF was variably present in both the stromal and tumor cell compartments. TF expressed by pericryptal sheath cells was progressively lost after the adenoma-to-carcinoma transition and was a strong predictor of survival in rectal but not colon cancer patients independently of disease stage, histological tumor grade and age.

In summary, this thesis demonstrates novel signaling mechanisms for the TF/FVIIa complex, and provides evidence of a hitherto unknown role of TF expressed by a specific population of stromal cells in colorectal cancer.

Keywords: Tissue Factor, blood coagulation, cell signaling, protease, mass spectrometry, immunohistochemistry, colorectal cancer, apoptosis, Eph receptor

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To Alfred

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Åberg, M., **Eriksson, O.**, Mokhtari, D., Siegbahn, A. (2014). Tissue factor/factor VIIa induces cell survival and gene transcription by transactivation of the Insulin-like growth factor 1 receptor. *Thrombosis and Haemostasis,* 111(4):748-60

II **Eriksson, O.**, Ramström, M., Hörnaeus, K., Bergquist, J., Mokhtari, D., Siegbahn, A. (2014) The Eph tyrosine kinase receptors EphB2 and EphA2 are novel proteolytic substrates of Tissue factor/coagulation factor VIIa. *Journal of Biological Chemistry,* 289(47):32379-91

III **Eriksson, O.**, Thulin, Å., Asplund, A., Hegde, G., Navani, S., Siegbahn, A. Tissue factor/coagulation factor VIIa potentiates ligand-dependent EphA2 signaling in cancer cells. *Manuscript.*

IV **Eriksson, O.**, Asplund, A., Hegde, G., Edqvist, PH., Navani, S., Pontén, F., Siegbahn, A. Tissue Factor in pericryptal sheath cells identifies a specific intestinal cell population and constitutes a candidate prognostic biomarker for rectal cancer. *Manuscript.*

Contents

Abbreviations

APC	Activated protein C
asTF	Alternatively spliced tissue factor
AT	Antithrombin
CAF	Cancer-associated fibroblast
CRC	Colorectal cancer
FFR-FVII	Active-site inhibited coagulation factor VII
FITC	Fluorescein isothiocyanate
FVII	Coagulation factor VII
FVIIa	Active coagulation factor VII
GPI	Glycophosphatidylinotisol
GTP	Guanosine triphosphate
HPA	Human protein atlas
IGF-1R	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
kDa	kiloDalton
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
PAR	Protease—activated receptor
PDGF(Rαβ)	Platelet-derreived growth factor (receptor alpha/beta)
PI3 kinase	Phosphatidylinotisol-3 kinase
PLA	Proximity ligation assay
PPP	Picropodophyllin
qPCR	Quantitative polymerase chain reaction
RTK	Receptor tyrosine kinase
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyachrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TRAIL	TNFα-related apoptosis-inducing ligand

Introduction

Blood coagulation is the process that protects us from bleeding and excessive blood loss upon an injury through the formation of a thrombus. Simultaneously, blood has to be kept soluble inside intact vessels and in order to achieve this balance a complex system involving circulating proteins and enzymes and blood cells has evolved. Moreover, thrombus formation is not the only consequence of activation of coagulation, and components of the coagulation system also directly act on cells and tissues to promote cellular activation and the inflammatory response. Thus, coagulation and inflammation are tightly linked, and integrated in the physiological response we mount to an injury. Likewise, excessive coagulation activation is involved in the pathogenesis of many of our common diseases. The molecular mechanisms behind these observations were studied in the present thesis, with a focus on the non-hemostatic properties of the coagulation system. In addition the clinical relevance of these findings were explored in a material of human colorectal cancer specimens.

Basic concepts in biochemistry and cell biology

Proteins are the macromolecules that carry out most of the work and tasks required for cells and tissues to function. They are built up from organic molecules called amino acids, which are assembled into proteins upon translation of mRNAs transcribed from protein-coding genes.

Proteins can be divided into broad classes according to their functions in the cell. Some examples include *structural proteins*, which support the shape and integrity of the cell, *enzymes* that catalyze chemical reactions and *signaling proteins*, including ligands and receptors, which allow cells to communicate with their environment and extracellular cues to be transmitted into the cell[1].

According to current estimates there are around 20 000 protein-coding genes in humans, but due to posttranscriptional and posttranslational modifications the actual number of possible protein isoforms are several magnitudes higher. Alternative splicing of mRNA transcripts can generate several protein isoforms from one mRNA species. Furthermore, enzymes catalyze a large variety of posttranslational modifications of proteins. *Covalent modifications*, exemplified by phophorylations or glycosylations, control the prop-

erties of a protein such as the activity of an enzyme and are frequently reversible. *Proteolytic cleavages* of peptide bonds are in contrast irreversible, and is either a non-specific event in protein degradation or in the case of limited proteolysis a regulatory mechanism that controls the activation state of proteins, as exemplified by e.g. coagulation factors. *Reduction of disulfide bonds* refers to the reductive cleavage of a covalent bond between two cysteine residues that couple their sulfur-containing side chains. Disulfides are mostly structural and not possible to modify, but a minority are *allosteric disulfides*, where a reductive cleavage is a means to alter protein function. As reduced disulfides can be re-oxidized, this modification is potentially reversible[2].

Cell migration

Cell migration refers to directional cellular movement and the translocation of a cell from one point to another[3]. Migration is induced by *chemoattractants*, extracellular substances that are present in a gradient which the cells migrate towards. To be able to respond to the chemoattractant, it must be sensed by the cell through the binding and activation of a receptor. Chemoattractants can either be soluble, in which case they induce *chemotaxis*, or components of the extracellular matrix, when directional migration is called *haptotaxis*. *Chemokinesis* refers to random movement in any direction, in contrast to chemotaxis which is directional. Cell migration occurs for example during embryonic development when tissues are formed and patterned, upon bacterial challenge when immune cells home to the site of infection, or when cancer cells spread and metastasize.

The cytoskeleton is a network of polymerized proteins found in all eukaryotic cells, which functions to give the cell its shape and to resist mechanical pressure. In human cells, it has the three main components *microfilaments* made of actin, *microtubules* consisting of tubulin, and *intermediate filaments* that have different components depending on cell type. The cytoskeleton is dynamic, and its ability to contract allows cells to move and migrate. Hence, the cytoskeleton must be targeted in signaling pathways that control these processes, where one important mechanism is the activation of the Rho family of small GTPases, a group of intracellular signaling molecules[4].

Apoptosis

Cell death can occur in two principally different ways[5]. The term *necrosis* is used for uncontrolled cell death, and may cause damage to neighboring cells through release of hazardous debris. Programmed cell death, or *apoptosis*, refers to a physiological event when unwanted cells are disposed of by the body in a controlled way. Triggers for apoptosis include factors that cause cellular stress such as radiation, nutrient deprivation and viral infections,

which activates the intrinsic pathway of apoptosis. Apoptosis can also be induced by extracellular ligands acting on death receptors, which is referred to as the extrinsic pathway of apoptosis. The apoptotic process occurs in a series of coordinated steps and has distinct morphological hallmarks such as cell shrinkage, blebbing and nuclear fragmentation. A family of cysteine proteases, *caspases*, has a central role in the apoptotic machinery. They are activated by proteolytic cleavages, and perform controlled proteolytical degradation of cellular components.

The coagulation system

As mentioned above, the coagulation system includes and is influenced by numerous components. These include cells such as platelets, white and red blood cells and the endothelium, circulating enzymes called coagulation factors and various activators and inhibitors. Coagulation factors are serine proteases mainly circulating in zymogen form, which are activated by a proteolytic cleavage. Coagulation factors are commonly denoted with a capital F and roman numerals, with the small letter a indicating their active form.

A complex sequence of event is required for the formation of a long-lasting thrombus and efficient sealing of a wound. When vascular integrity is disrupted circulating platelets will immediately adhere to the site of inury, mediated by interactions between platelet receptors and the endothelium, e.g. through the cross binding of the von Willebrand factor between platelets and collagen. As a result of their adhesion, platelets will become activated and release the pro-coagulant contents from their intracellular granulae. In addition, platelets aggregate by binding to each other, e.g by cross-binding of fibrinogen through the GPIIbIIIa receptor, and a platelet plug is formed,

For the platelet plug to be stabilized, a protective fibrin meshwork needs to be formed, which is accomplished in an amplification reaction where coagulation factors activate each other. Concurrently with platelet adhesion, the transmembrane protein Tissue Factor (TF), will be exposed to blood and bind its ligand coagulation factor VII or its active form VIIa. The resulting Tissue Factor/factor VIIa (TF/FVIIa) complex activates factors IX and X, which leads to generation of small amounts of thrombin (also called factor II). Thrombin contributes to further platelet activation and generates local increase in active forms of other coagulation factors, such as FV and FVIII released from platelet granulae. The process then moves to the surfaces of activated platelets, which provide a negatively charged surface that is rich in phosphatidylserine and decorated by activated co-factors. On the platelet surface, activated FIX associates with its co-factor FV, forming the “tenase complex” which converts zymogen FX to its active form. Finally FXa together with FVa form the “prothrombinase complex” which generates large amounts of activated thrombin, with concentrations now far exceeding those

obtained initially by the TF/FVIIa complex. Active thrombin then converts fibrinogen to fibrin monomers, which forms a fibrin meshwork that stabilizes the platelet plug into a thrombus[6,7] (Fig. 1).

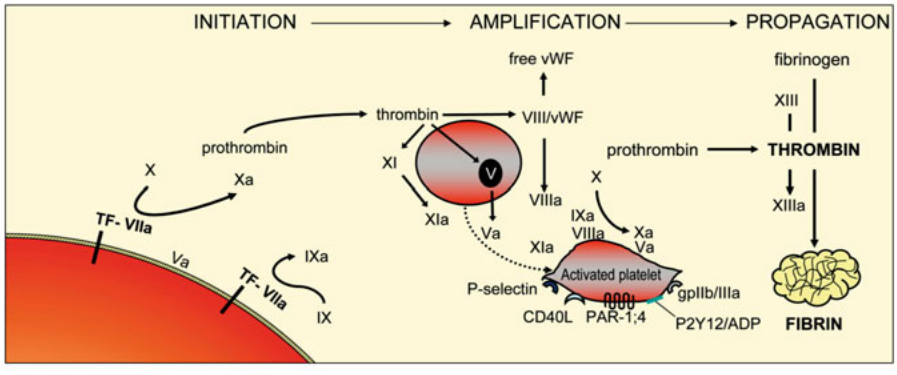

Figure 1. The cell-based model of coagulation. Image created by Mikael Åberg and Agneta Siegbahn.

Regulation of the coagulation process

Since aberrant intravascular coagulation activation would be hazardous, several mechanisms exist to keep the coagulation process tightly controlled. The fibrin meshwork is degraded during *fibrinolysis* by the proteolytic enzyme plasmin, which serves to limit thrombus expansion and remove the clot once the wound is healed. Circulating *protease inhibitors* inactivate coagulation factors that diffuse away from the wound and keep the process localized. Specific inhibitors to one or several coagulation components have evolved, exemplified by *antithrombin (AT)* which inhibits e.g. FXa and thrombin in a heparane sulfate dependent reaction, *activated protein C (APC)* which in complex with protein S inactives FVa and FVIII and *Tissue Factor Pathway inhibitor (TFPI)* which acts on the TF-dependent initiation phase. However, during inflammation and disease these inhibitor systems may be compromised or down-regulated contributing to the pro-coagulant state observed in many conditions.

Tissue Factor

Tissue Factor (TF, alternative names F3, CD142 or thromboplastin) is a 47 kDa transmembrane protein functioning as the physiological initiator of blood coagulation[8]. TF is related to the cytokine receptor family, but functions have diverged considerably throughout evolution and the relationship

in humans is believed to be mostly structural[9]. TF is a 263 amino acid protein with a large extracellular part with a binding site for FVII/FVIIa, a small transmembrane part, and a 21 amino acid cytoplasmic tail with three potential serine phosphorylation sites. The extracellular domain of TF contains 4 cysteine residues that form two disulfide bonds (Cys49-Cys57 and Cys186-Cys209) (Fig. 2). TF is transcribed from the F3 gene on chromosome 1, and is composed of 6 exons, where exons 1-5 correspond to the extracellular part and exon 6 the transmembrane part and intracellular tail of TF[10]. Apart from full length membrane bound TF, alternative splicing generates an mRNA where exon 5 is skipped. This leads to a frame shift, and the new transcript produces a protein with a unique C-terminus called alternatively spliced TF (asTF) that is soluble instead of anchored in the cell membrane[11]. asTF is by most accounts not procoagulant, but posses non-hemostatic functions and potently stimulates angiogenesis[12].

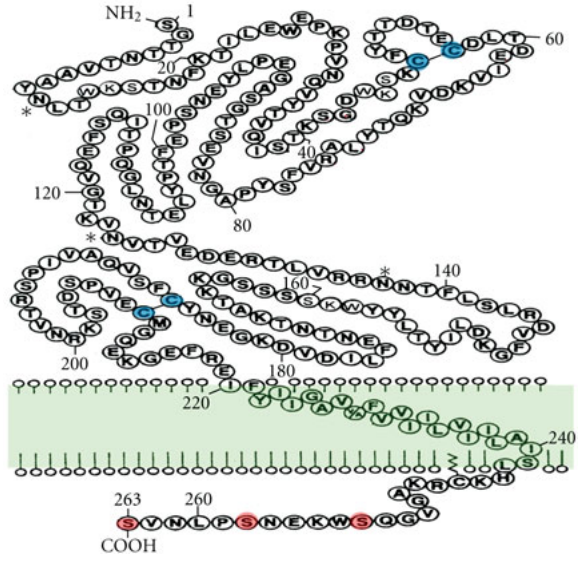

Figure 2. The structure of TF. The two disulfide bonds in the extracellular domain are indicated in blue, and the three serine residues in the cytoplasmic domain in red. Adapted from Chu AJ, Int. J Inflammation, 2011.

Sources of TF

TF shows a variable expression pattern, with constitutive expression in squamous and respiratory epithelia, the gastrointestinal tract and the adventitia of larger blood vessels. Notably, high TF expression is found in locations where a bleeding would be fatal, such as the brain and placenta (Fig.3). While TF expression is prominent in extravascular tissues it is normally low or absent inside vessels, an observation that gave rise to the concept of a

"hemostatic envelope", meaning that TF acts as a barrier ready to activate coagulation when vascular integrity is disrupted[13].

However, several sources of inducible intravascular TF exist, as a means to promote thrombus formation and inflammation. It has long been recognized that monocytes show inducible TF expression after exposure to bacterial Lipopolysaccharide or pro-inflammatory cytokines such as MCP-1 or PDGF-BB[14]. In addition, many cell types including blood cells release microparticles upon activation, which are small (diameter 0.1-1 μm) corpuscles surrounded by a functional plasma membrane. Microparticles from activated monocytes contain phosphatidylserine on their surface and may be rich sources in TF, thus contributing to the blood-borne TF pool. TF expression in other blood cells than monocytes is an ongoing controversy, and evidence both in favor of and against TF expression in neutrophils, eosinophils and thrombocytes have been presented[15]. Although TF may be found associated with e.g. platelets during pro-inflammatory and pro-thrombotic conditions, it remains unclear wheter TF is actually synthesized and expressed by these cells, as a likely alternative is that monocyte-derived microparticles associated with platelets is the origin of this source of TF[15].

Thus during active inflammation monocytes provide a pro-coagulant surface inside the bloodstream, a mechanism that may contribute to the excessive and uncontrolled coagulation activity in severe forms of sepsis and disseminated intravascular coagulation. Likewise physiological reasons for intravascular TF expression have been proposed, both an integral role in normal thrombus formation[16], and a function in defense against pathogens as thrombus formation in small capillaries could prevent invasion of bacteria into tissues[17]. Additionally, as described in more detail below, not all blood-borne TF is believed to be pro-coagulant unless it is activated, which explains why blood-borne TF can be present in healthy individuals without uncontrolled thrombus formation.

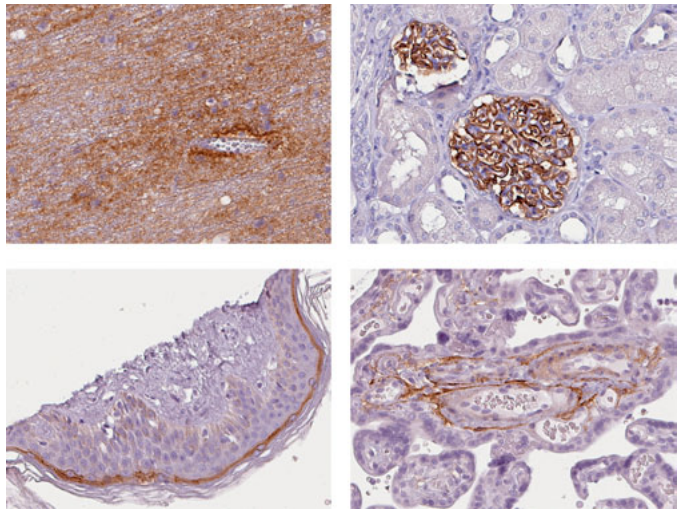

Figure 3. TF expression in human tissues. Immunohistochemistry micrographs showing clockwise from upper row: brain, kidney, placenta and epidermis. TF is visible as brown staining. The images were generated using a polyclonal anti-TF antibody developed by the Human Protein Atlas project.

Coagulation factor VII/VIIa

Coagulation factor VII (FVII/FVIIa) is synthesized in the liver as a single chain protein of about 50 kDa, and posttranslationally modified by N-linked glycosylations and gamma-carboxylation on its light chain in a vitamin K-dependent reaction. TF is the essential co-factor and receptor for FVIIa, which does not contain significant catalytic activity on its own. TF serves to localize FVIIa to the cell membrane, and in addition stabilizes the FVIIa active conformation[18]. FVII is converted from the zymogen form to the active FVIIa form by a proteolytical cleavage at the arginine-152-isoleucine-153 bond to form a molecule consisting of a light chain and protease-domain containing heavy chain held together by a single disulfide bond. Activation is performed by a number of coagulant or non-coagulant proteases, including FX, FIX and TF/FVIIa itself[19].

FVII circulates mainly in zymogen form, and its plasma concentration has been estimated to to 470 ± 112 ng/ml or, assuming a FVII molecular weight of 50 kDa, to 9.4 ± 2.24 nM in healthy individuals[20]. A small fraction of circulating FVII is present in the enzymatically active form FVIIa, and a study using a TF mutant that selectively binds FVIIa estimated the plasma concentrations of FVIIa in healthy individuals to 3.58 ± 1.44 ng/ml[21]. This gives a molar concentration of 0.072 ± 0.029 nM, which equals 0.076 % of the total plasma FVII concentration. However, another report using an ELISA assay based on an antibody selectively recognizing FVIIa suggested the

concentrations of FVIIa may be even lower, since artefactual activation may lead to overestimations in the TF mutant assay[22]. Based on these and other studies, a FVIIa concentration of 10 nM is, somewhat contradictory, commonly used experimentally as a physiological concentration. However, although this is far from the circulating levels of active FVII it is assumed that zymogen FVII is rapidly activated upon binding to TF.

FVIIa is, like other coagulation proteases, a tryptic serine protease with the characteristic Ser-His-Asp triad in the catalytic site[23]. Trypsin-like serine proteases cleave substrates at peptide bonds following a positively charged amino acid such as arginine or lysine, which interacts with a negatively charged amino acid in the enzyme. FVIIa appears to have rather narrow cleavage specificity as only a few substrates are known, in total including FX, FIX, TF/FVIIa itself[24] and PAR2[25,26]. These substrates have in common an arginine residue at the P1 position at the cleavage site (Fig 4). That is a general requirement of coagulation proteases, yet FVIIa has a more narrow cleavage specificity than e.g. thrombin[27] which underscores that other substrate characteristics in addition to the primary amino acid sequence determines if a proteolytic cleavage will occur. For FVIIa these remain incompletely understood, but will likely include general determinants of limited proteolysis such as accessibility and secondary structure[28].

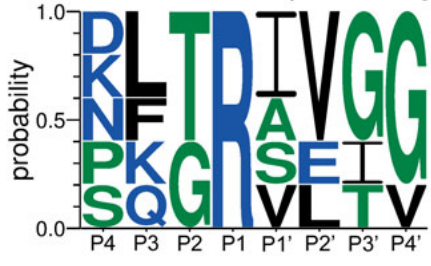

Figure 4. Amino acid sequence logo showing the consensus sequence at the cleavage sites of the FVIIa substrates FX, FIX, FVII and PAR2. Colors indicate amino acid hydrophobicity. The figure was generated using the WebLogo 3 application.

The concept of cryptic TF

Early on in studies on TF it was discovered that only a fraction of cell surface TF was able to support FX activation and coagulation initiation[29]. Since all available TF at the cell surface bound FVIIa, the concept of two different cellular TF pools, termed cryptic and active TF, was proposed. The classic definition of cryptic TF indicates a TF molecule that binds FVIIa, but the resulting TF/FVIIa complex fails to activate the macromolecular substrates FX and FIX[30]. However, cryptic and active TF also differ in their interaction with FVIIa. Although both TF pools readily form the TF/FVIIa complex, it

appears that active TF binds FVIIa more rapidy and with higher affinity[31]. Commonly, FX activation by TF expressing cells are saturated at low concentrations when FVIIa is bound to only a small fraction of available TF, with K_D values in the subnanomolar range. In contrast, higher FVIIa concentrations are needed to saturate all TF binding sites, which nonetheless appears to occur below the plasma concentration of total FVII after prolonged incubations[32].

TF decryption/encryption has been proposed as a regulatory mechanism to control TF procoagulant activity, in order to keep blood-borne TF inactive during resting conditions and to avoid unwanted intravascular coaguation[29]. The exact mechanism for TF decryption/encryption is at present controversial. It is generally accepted that negatively charged phospholipids in the cell membrane play a major role. Interestingly, the major contribution of these appears to no be in facilitating docking of the TF/FVIIa substrates, but rather in inducing conformational changes in TF that exposes substrate binding sites for FX and FIX[30]. Another theory for regulation of TF activity concerns the redox status of the TF Cys186-Cys209 disulfide bond and its regulation by protein disulfide isomerase. Mutational studies has revealed that an intact (i.e. oxidized) Cys186-Cys209 disulfide is required for TF/FVIIa catalytic activity towards FX, whereas it has been suggested to be reduced in cryptic TF[33,34,35]. Thus it has been proposed that the Cys186-Cys209 disulfide is an allosteric one, i.e. a disulfide that is dynamically reduced and oxidized and thereby controls the ability of TF to support coagulation activation. However, this mechanism have been questioned[36], and the Cys186-Cys209 disulfide has not yet been demonstrated in reduced form *in vivo* using direct quantitative methods such as mass spectrometry. Moreover, although the role of disulfide bond modification by protein disulfide isomerase released from plateles and endothelial cells is well established during thrombus formation in general, its role in promoting TF oxidation in this context remains controversial[37].

TF signaling

In addition to its role as the trigger of blood coagulation, TF together with its ligand FVII/FVIIa functions as a true signaling receptor, both on its own and through cross-talk with cell-surface receptors. Apart from direct activation of cell signaling by the binary TF/FVIIa complex, activation of coagulation by TF/FVIIa generates active downstream coagulation proteases, which in turn are potent signaling molecules.

Protease-activated receptors.

The cloning of the thrombin receptor in 1991 marked the discovery of a new family of G protein coupled receptors, characterized by a proteolytical cleavage as the activation mechanism[38]. Termed Protease-activated receptors (PARs), these are cleaved by extracellular proteases near the N-terminus on the luminal face of the cell membrane. The cleavage exposes a novel N-terminus which functions as a tethered ligand that folds backs to and activates the receptor. Based on the novel N-terminal activation sequence, artificial PAR agonists can be synthesized. In humans, 4 PARs (PAR1-4) have been described to date, and they constitute the prototypical signaling receptors for coagulation proteases. PAR1 is the main thrombin receptor, but thrombin also cleaves and activates PAR3 and PAR4. Subsequently, a thrombin-insensitive PAR, PAR2, was discovered[39], which was later found to be activated by TF/FVIIa[25].

PARs are not exclusively cleaved by coagulation factors, but are activated by a number of proteases and can be described as cellular protease sensors allowing cells to detect and respond to an increase in extracellular protease activity. In the PAR2 case, trypsin and mast cell tryptase are potent activators with EC_{50} values around 1 nM[40]. Kallikreins, a large protease family involved in inflammation and epithelial homeostasis also activate the PARs[41]. The canonical cleavage site of all four PARs is located after an arginine residue indicating that they are foremost activated by trypsin-like serine proteases. Recently, biased PAR signaling has been acknowledged, where PARs are cleaved at a site distinct from the canonical cleavage site by additional proteases such as matrix metalloproteases, with differential downstream signaling response as a result[42].

Mechanisms of TF signaling

PAR2 activation by TF/FVIIa initiates a pro-inflammatory and pro-angiogenic cellular program, characterized by secretion of cytokines and angiogenic factors, and increased cell motility[43,44]. Likewise, TF/FVIIa-PAR2 signaling has been shown to activate the major cellular signaling nodes such as the ERK and PI3K pathways, although most of these data is derived from cancer cells and immortalized cell lines[45].

In contrast to FX activation, both the active and cryptic cellular TF pools support PAR2 cleavage by FVIIa, and relatively high FVIIa concentrations are needed to activate PAR2 signaling. In the commonly used MDA-MB-231 breast cancer cell line with high endogenous expression of TF and PAR2 the EC_{50} values for PAR2 induced gene transcription were around 5 nM FVIIa[46], compared to the subnanomolar values required for FX activation discussed above. The fact that relatively high FVIIa concentrations are needed for PAR2 activation raises the question about a physiological rele-

vance of these findings. However, these values are still below the plasma concentration of total FVII, and animal models have provided evidence for a role of TF/FVIIa/PAR2 signaling in cancer development and obesity[47,48].

Apart from direct PAR2 activation by the binary TF/FVIIa complex, TF/FVIIa indirectly supports signaling through other PARs. The ternary TF/FVIIa/FXa complex efficiently activates PAR1 and PAR2[49], through a mechanism supported by the endothelial protein C receptor[50]. Through activation of thrombin, TF/FVIIa contributes to thrombin-dependent activation of PAR1.

The TF cytoplasmic domain is dispensable for coagulation activation and some aspects of PAR2-signaling, but can be phosphorylated on serine residues and control incorporation of TF into microparticles[51] and integrin-dependent adhesion and cell migration[52]. Although TF/FVIIa is one of many activators of PAR2, an important role for TF in PAR2 signaling is suggested by the fact that it is thought to be regulated by the TF cytoplasmic domain. It was shown to exert negative regulatory control on PAR2, which was released upon PAR2-dependent serine phosphorylation of the TF cytoplasmic domain[53].

TF associates with cell surface integrins, an association that is constitutive on cancer cells, and enhanced by FVII/FVIIa binding on non malignant cells[47]. Interactions between TF and integrins support cell spreading and migration in some contexts[52], and a recent study demonstrated that the interaction of β1-integrin with TF functions potently in inducing angiogenesis[54]. The alternatively spliced TF isoform, asTF, is not capable of PAR2 activation but signals through integrin ligation to support angiogenesis[12].

TF and cell migration

The TF/FVIIa complex is closely connected to mechanisms that control cell motility. FVIIa can act as a chemoattractant on its own, which was shown to be mediated by PAR2-dependent autocrine IL8 production in MDA-MB-231 breast cancer cells[55]. Furthermore TF/FVIIa/PAR2 signaling sensitizes smooth muscle cells and monocytes to PDGF-BB-mediated cell migration, which is triggered at 100-fold lower PDGF-BB concentrations when FVIIa is present[56,57]. In addition, FVIIa potentiates PDGF-BB mediated angiogenesis[53] and the use of cell lines and mice with a deleted TF cytoplasmic domain has shown its involvement in these processes[58].

Recent studies using vascular smooth muscle cells and endothelial cells have highlighted the role of TF in migration of these cell types during blood vessel formation through a mechanism involving CCL2 production by endothelial cells[59]. Although FVIIa ligation by TF sensitizes vascular smooth muscle cells to PDGF-BB, it was also shown that TF silencing impairs PDGF-BB stimulated migration independently of addition of FVIIa[60]. Fur-

thermore, TF localized to the leading edge of migrating cells together with PAR2 and filamin.

TF and apoptosis

The TF/FVIIa complex also promotes cell survival, through reduction of caspase activation induced by serum starvation or death receptor ligands[61,62]. Both PAR1 and PAR2 activation was excluded in the context of antiapoptotic signaling by TF/FVIIa, showing that TF/FVIIa can signal independently of PARs.

Cancer and the coagulation system

Cancer is associated with a pro-coagulant state and an increased risk of thrombosis, an observation that was made by the French physician Trousseau already in the 19th century. Since then, epidemiological studies have shown that patients with an idiopathic venous thrombosis have an increased risk of developing an overt cancer within the near future[63], and that patients with cancers complicated by thrombosis have an increased risk of developing distant metastases[64]. The sources of the pro-coagulant state observed in cancer patients likely includes a combination of systemic cytokine effects, pro-coagulant microparticles released from cancer cells and TF expressed by cancer cells themselves or circulating microparticles[65]. These studies underscore the relationship between coagulation and cancer, but do not answer the question whether activation of coagulation directly promotes tumor progression, or merely is a consequence of a disseminated cancer. Although anti-coagulant treatment strongly reduces cancer progression in some studies on mice, anti-coagulant treatment in humans only has modest, if any, effects on survival[66,67]. Moreover, studies on cancer incidence in long term users of the anti-coagulant warfarin has generated conflicting results on a possible cancer preventive effect, although a small protective effect on the development of prostate cancer appears to be consistent[68,69]

TF expression by tumor cells was first systematically evaluated in a study published in 1992, where some degree of TF positivity was found in most solid tumor types using immunohistochemistry (IHC)[70]. These findings have then been extended by independent groups, demonstrating that TF positivity is correlated with tumor grade and disease stage in e. g. cancers of colorectal[71], breast[72] and central nervous system origin[73]. However, IHC remains an application that is crucially dependent on antibody quality, and many of these studies did not include antibody validation for IHC.

In contrast to conflicting clinical and observational data, a number of experimental studies support an important role for coagulation and TF/FVIIa signaling in cancer progression. Studies using TF expressing cancer cell

lines have demonstrated that TF modulates various aspects of cell behavior such as cell migration and invasion[55], and resistance to apoptosis[62].

Initial studies on the role of TF in hematogenous metastasis in immunodeficient mice demonstrated a strong enhancement of lung colonization in malignant cells overexpressing TF, which was lost in mutants with a deleted cytoplasmic domain[74]. Later studies in immunocompetent mice confirmed the requirement of TF in hematogenous metastasis, but failed to reproduce to role of the cytoplasmic domain. Instead, in these models TF mediated coagulation enhanced survival of micrometastases through protection from NK cell eradication and enhanced monocyte recruitment[75,76]. Thus it appears that TF mediated coagulation, rather than non-coagualant signaling supports these processes. Mouse models have instead suggested a role for the TF/FVIIa-PAR2 axis in primary tumor growth[47].

Signaling by receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are a class of cell surface receptors for extracellular ligands which are characterized by their intrinsic tyrosine kinase activity. Ligand binding induces dimerization of receptor subunits and activation of the tyrosine kinase moiety, and transphosphorylation of tyrosine residues in the cytoplasmic domain of the receptor follows. These tyrosines provide binding sites for SH2- and PTB-domain-containing adapter proteins that initiate downstream signaling transduction through e.g. the ras/MAPK or PI3K/Akt pathways. RTK ligands include growth factors and hormones, substances necessary for cellular proliferation, growth and motility. RTK signaling is vital for the proper growth and functions of cells, and has also a central role in the pathogenesis of many human diseases. Deregulated growth factor signaling is a central event in many cancers and drives the uncontrolled proliferation of malignant cells. Given its potent effects, RTK signaling kept in tight control at several levels[1].

Modulation of RTK signalling

Receptor downregulation and endocytosis follows activation in a feedback-like manner to terminate signaling, and tyrosine kinase activity is controlled by cytoplasmic protein tyrosine phosphatases (PTPs). RTK activation can also occur in the absence of its cognate ligand, by a transactivation[77]. RTK transactivation was originally described in studies where GPCR agonists induced activation of the EGF receptor (EGFR)[78] and the EGFR has been the model receptor for transactivation studies since. At least two mechanisms have been identified, one including release of a membrane bound form of EGF, and the other an intracellular pathway mediated by proteins of the Src family. These studies have been extended to other RTKs as well, and it has

become clear that most RTKs are probably activated by GPCR agonists in one or more of these ways, thus transactivation provides one of several means for GPCRs to control cell proliferation, migration and apoptosis. Thus, the view of an RTK as a static molecule in the cell membrane responding to extracellular ligands does not cover all aspects of RTK signaling. Apart from transactivation, RTK function is frequently modulated by proteolytic cleavages at the cell membrane[79], and signaling frequently continues even after endocytosis of an RTK and its removal from the plasma membrane. It has also become evident that RTKs can translocate to the nucleus and directly control gene expression[80].

Receptor tyrosine kinases have evolved to include a large number of subfamilies in humans, of which two were studied more closely in this work.

IGF-1R signaling

The insulin and IGF-1 receptor families share a common ancestor and still have considerable homology. Their functions have converged through evolution where the insulin receptor has come to regulate carbohydrate metabolism and IGF-1R proliferation and cell growth[81]. The IGF-1R is activated by its ligands IGF-1 and IGF-2, and at high concentrations, insulin. IGF-1 is synthesized in the liver in response to growth hormone released from the pituitary gland or locally in tissues, so in contrast to most other RTK ligands, IGF-1 acts not only in a paracrine fashion but also systemically as a hormone to stimulate proliferation and growth. IGF-1R is expressed on most cells in the body and often expressed by neoplastic cell lines and human cancers[82]. Structurally, IGF-1R is a tetramer consisting of two α-subunits and two β-subunits, held together by disulfide bonds. Ligand binding to the α-subunit of the receptor triggers autophosphorylation of the three tyrosine residues, Tyr1131, Tyr1135, Tyr1136 in the activation loop within the kinase domain of the β-subunit. Phosphorylation of other residues in the β-subunit serve as docking sites for Insulin receptor substrate (IRS) proteins among others, mediating the signalling cascades induced by IGF-1 stimulation[83]. Additionally, three C-terminal serine residues were found to proved binding sites for adapter proteins of the 14-3-3 family[84]. Recently, the intact IGF-1R was shown to translocate to the nucleus in tumor cells. IGF-1R was bound either directly or in a complex to DNA, indicating that it controls gene expression. Posttranslational modification by SUMOylation of three lysine residues in the β-subunit of IGF-1R was suggested as imperative for the nuclear translocation described in this paper[80].

Cells receive a multitude of anti-apoptotic cues from the environment, many of which converges on the IGF-1R. Substances ranging from ECM components such as fibronectin[85] to $GABA_B$ receptor agonists in the nervous system[86] are depending on the IGF-1R to promote cell survival, highlighting the role of IGF-1R in protection from apoptosis. IGF-1R signaling has also

caught recent attention as a target for therapy in human cancer, where interference with IGF-1R is anticipated to reduce tumor cell survival[83].

Eph receptor signalling

The Eph tyrosine kinase receptors constitute with 14 members the largest RTK family in the human genome. The Eph structure includes an extracellular part with a ligand-binding domain, a Cys-rich domain mediating lateral interactions with other Eph receptors and two Fibronectin type III repeats. The intracellular part contains a kinase domain which is autophosphorylated upon ligand binding, and a sterile α motif and a C-terminal PDZ domain mediating additional interactions with intracellular proteins[87]. A unique feature of Eph signaling is that the ligands, called ephrins, are membrane bound and that signaling preferentially occurs at cell-cell contacts. The Eph-ephrin interaction generates a signal both into the Eph expressing cell through receptor autophosphorylation ("forward signaling") as well into the ephrin expressing cell ("reverse signaling"). Eph receptors and ephrins are divided into A and B classes on basis of receptor-ligand affinities, where five EphB receptors (EphB1-4 and EphB6) bind B-class transmembrane ephrins while nine EphA receptors (EphA1-8 and EphA10) preferentially bind GPI-anchored ephrin-A ligands (Fig. 5).

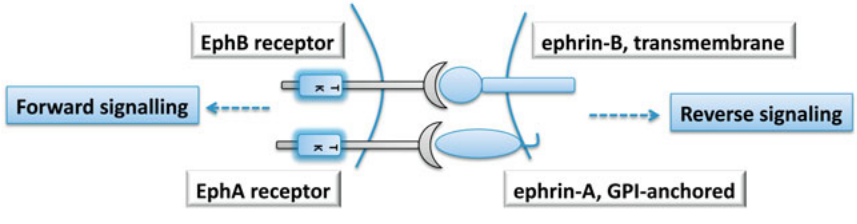

Figure 5. Schematic overview over Eph-ephrin signaling.

Compared to other RTKs, Ephs need not only to dimerize to become active, but higher order clustering of multimeric complexes on the cell surface is required for a maximal signaling response[88]. This is mediated by ephrin ligands expressed on the opposing cell, suggesting that the main role of ephrin ligands seems to be to increase the local concentration of receptors so that efficient multi-order clustering can occur. Eph receptors are also capable of a certain degree of clustering without ligand-mediated activation[89]. Interactions between Eph receptors *in cis* between the ligand-binding and Cys-rich domains or the ligand-binding and fibronectin type III repeat domains have been described for several Ephs. This pre-clustering of Eph receptors is

thought to promote fast and efficient receptor activation upon binding of ephrin ligands.

Signaling between Eph and ephrin expressing cells often results in repulsive responses with cell rounding and loss of focal adhesions as a consequence[90], but signals may also be converted to adhesion and increased cell migration[91]. The relative abundances of different Ephs and ephrins in the signaling clusters have been proposed to determine the outcome, which may explain why seemingly opposite functions are recorded in different experimental systems[92]. Acting as global cell positioning system, Eph-ephrin signaling control cell positioning and tissue homeostasis, and play important roles in embryonic development, organization of the nervous system and angiogenesis where they function as a guidance system.[93,87,94]. Its role in maintenance and development of the intestinal epithelium is very illustrative of the their functions in cell positioning and tissue organization[95]. In intestinal epithelial cells, EphB receptors are highly expressed at the bottom of the crypts with a decreasing gradient upwards. In contrast, the ephrin-B-ligands are expressed in the apical portions of the glands but absent in the crypts. Studies on mice have demonstrated that, in line with this expression pattern, repulsive EphB-ephrin-B signaling controls cell positioning in the intestinal epithelium. EphB positive intestinal stem cells and Paneth cells are confined to the crypt bottom through repulsive ephrin-B signals. As they differentiate, they lose their EphB expression and can move upwards in the epithelium.

At cell-cell contacts Eph and ephrins form high-affinity interactions in multivalent clusters that constitute a sort of intercellular tethers keeping cells tightly attached. Yet, a common outcome of the Eph-ephrin interaction is cell detachment and repulsion. For this to be achieved the Eph-ephrin interaction has to be terminated and a number of mechanisms regulating this process have been proposed. Especially much attention has been focused on the role of extracellular proteases, where matrix metalloproteases (MMPs), members of the disintegrin and metalloprotease (ADAM) family and neuropsin have been implicated in regulating Eph function[96]. A well-characterized mechanism has been described for the ADAM10 protease in regulation of EphA3 signaling. ADAM10 is constitutively associated with EphA3 but is not proteolytically active. However, ligation of EphA3 with its ligand ephrin-A5 creates a new protease recognition site in ephrin-A5, leading to its cleavage by ADAM10 and termination of the signaling interaction by a mechanism that ensures that only receptor-bound ephrin-A5 is cleaved[97].

The Eph-ephrin system in cancer

In line with a function in maintaining tissue organization and homeostasis the Eph-ephrin system is frequently dysregulated in cancer[98], and Eph receptors have been characterized as both tumor suppressors and oncogenes depending on cancer type and the experimental model used. Ligand-dependent

signaling appears to be tumor-suppressive in some contexts, by exerting inhibitory effects on cell migration and motility. EphA2 of the EphA subclass was shown to be an effector for PI3 kinase / Akt signaling though serine phosphorylation on its cytoplasmic domain with important effects on cell invasion[99] and cancer stem cell maintenance in glioblastoma[100]. These processes were counteracted by the EphA2 ligand ephrin-A1, demonstrating opposite effects of ligand-dependent and ligand-independent Eph signaling.

However, recent studies have indicated that malignant cells respond differently to ephrin ligands, suggesting that ligand-induced Eph activation can also be tumor promoting. Specifically, a number of publications have highlighted the role of EphA2-ephrin-A1 signaling and downstream RhoA activation in cancer cells with the acquisition of a rounded cellular phenotype and the transition from collective to a type of single cell invasion termed amoeboid invasion[101,102]. Eph-ephrin interactions mediating contact-inhibition of locomotion between cancer cells have also been proposed to increase cellular dispersion from the main tumor mass and facilitate cancer dissemination[103].

The Human Protein Atlas

The human Protein Atlas (HPA) is a proteomic effort aiming at developing a map of protein and gene expression in human tissues using RNA sequencing and IHC[104]. The output is a publicly available atlas published on the internet (www.proteinatlas.org) including transcript data and annotated IHC images. Antibodies for IHC are generated in-house by a large scale effort, and in the final release of the atlas IHC images based on more than 24 000 antibodies are included. The antibody production and validation pipeline includes bioinformatic selection of immunogen sequences, immunization of rabbits to raise polyclonal antibodies and evaluation of staining patters on human tissues in relation to published data and relative RNA levels. As a further validation tool IHC on cell lines is used, where correlation of antibody staining with relative RNA levels provide a good measure of antibody specificity[105].

Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer, and a leading cause of death world-wide[106]. Surgery is the primary curative treatment, and can be combined with adjuvant chemo- or radiotherapy in different patient subgroups. CRC comprises cancers originating in the colon and rectum, but tumors from these two locations display some differences. Treatment protocols and prognosis differ, and some studies have suggested biological differences in rectal and colonic tumors as well[107]. Like cancer in general, CRC is

classified into disease stages based on the extent of primary tumor growth and the presence of metastases to lymph nodes or distant sites. After surgery, the tumor specimen is subjected to pathological examination which classifies the tumor according to its histology and growth pattern. In addition, expression studies of certain proteins and/or mutational analysis are performed to further grade the tumor and provide additional prognostic information. Disease stage is the main prognosticator in use clinically, where stage II (no lymph node involvement) confers a good prognosis with around 60-85% 5-year survival in comparison with stage III (lymph node metastases present) that has a 45-65% 5-year survival. The search for additional biomarkers is ongoing, although very few have made it to clinical use so far.

Current investigations

Methods

Cell culture

Cell culture refers to the growing and cultivation of cells in the laboratory, and has become an indispensable tool in research as a model system to study cellular function in health or disease. A number of cell lines were used in this thesis to study TF signaling. The metastatic MDA-MB-231 breast cancer cell line was used in papers I-III as a general model system. This cell line expresses very high levels of TF and is commonly used in the field to study TF function and signaling. Metastatic PC3 prostate cancer cells were used in paper I to study the effects of FVIIa on apoptosis, and U251 glioblastoma cells were used in papers II-III for studies on Eph-receptor signaling. Experiments were also performed on primary cells, which have not been transformed or immortalized and have a limited life span *in vitro*. Freshly isolated monocytes were used in paper I. If derived from healthy individuals these cell do not express TF, therefore expression is induced after isolation by Lipo-polysaccharide treatment. Primary human fibroblasts were obtained from foreskin resections of newborns, and constitutively express TF under cell culture conditions.

Methods to study proteins

Biological samples are often very complex and consists of a multitude of proteins and other macromolecules. Examples of such samples are body fluids, or cell or tissue lysates. To reduce the complexity of a sample, it can be separated by electrophoresis exploiting the differential electrophoretic mobility of its constituents. A current is applied to a sample in solution, where macromolecules will migrate according to their charge-to-mass ratio. For separation of proteins, polyacrylamide gels are used, which from a three-dimensional meshwork with pores through which the proteins migrate, where smaller proteins migrate faster than larger ones. By the use of the anionic detergent sodium-dodechyl sulfate (SDS) which denatures the proteins resulting in similar charge:mass ratios, the molecular mass of a protein will be the main determinant of gel migration and thus the molecular weight of a protein can be estimated based on the migration distance

through the gel. This technique is called *SDS-Polyachrylamide gel electrophoresis*, or SDS-PAGE.

Western blot

A cornerstone method in protein studies, Western blot combines SDS-PAGE with blotting of the separated proteins onto a membrane and immunodetection using antibodies. Secondary antibodies conjugated to e.g. the enzyme horse-radish peroxidase or fluorophores are used for detection. Western blot gives information about the size of the protein of interest, and provides an estimate of the relative quantity of a protein. Using e.g. phosphospecific antibodies, post-translational modifications of proteins can be studied. Western blot was used in papers I-III of this thesis.

Imaging of cells and proteins

Cells are too small to be seen by the naked eye, but can be visualized by microscopy which magnifies the view of the object one is studying. In light microscopy, visible light and a system of lenses are used to magnify the object. Fluorescence microscopy uses fluorescence to generate an image of the object, which is labeled by an antibody or a probe conjugated to a fluorescent dye. By using different fluorophores several proteins can be studied in one sample. Confocal microscopy is a development of basic epifluorescence microscopy, which uses point illumination of the sample, and a pinhole to eliminate out of focus light. As a result, a confocal micrograph represents only a thin slice of the object, but 3D images can be reconstructed from single micrographs taken at the different planes in the object.

Fluorescence microscopy was used in papers I-III in this thesis, and light microscopy was used in papers III-IV to capture images of IHC stained tissue sections.

Proximity ligation assay

Originally devised and developed in Uppsala, Sweden, the proximity ligation assay (PLA) is an antibody based protein detection method that requires dual recognition of the antigen by two different antibodies, and translates protein recognition by antibodies into detection of a PCR product[108]. The assay uses secondary antibodies coupled to oligonucleotides, which provide a template for a PCR-based rolling circle amplification reaction only when two antibodies are in close proximity. The resulting PCR product is hybridized to fluorescently labeled DNA probes, which can be quantified. *In situ* PLA is performed on cultured cells or tissue sections, where the PLA signal is visualized as red fluorescent dots by fluorescence microscopy and can be counted for quantification. Depending on which primary antibodies are used, the PLA method allows for detection of proteins, complexes of interacting proteins or post-translational modifications

Immunohistochemistry (IHC)

IHC refers to antibody-based stainings of intact tissues, and is widely used for studies of proteins in a tissue context. Tissue material can be prepared either fresh frozen, or formalin-fixed and paraffin-embedded which allows long term storage. The tissue specimen to be studied is sliced to sections a few micrometers thick and mounted on to glass slides, where antibody staining is performed. Most commonly immunoperoxidase staining is used for detection in IHC, using secondary antibodies conjugated to a peroxidase enzyme that catalyzes a color-producing reaction, but IHC can also be combined with immunofluorescence. To enable enhanced visualization of the tissue components, tissue sections are counterstained by defined solutions, such as Mayer's hematoxylin solution. When using immunoperoxidase staining, tissue sections are examined using light microscopy.

IHC is, like any antibody-based analytical method, highly dependent on antibody quality. The suitability of an antibody for one method, e.g Western blot, does not guarantee its reliability in another method such as IHC, since intact tissues may compromise recognition of the target epitope or lead to non-specific reactivity to other proteins. Moreover, in Western blot proteins are separated according to size, which will aid in the assessment of the specificity of an antibody signal. This is not possible using IF or IHC, which complicates the evaluation of antibody specificity. When judging antibody performance, antibody reliability can be estimated by comparing the results to previously published data on the expression and localization of the target protein or, ideally, by comparison with the staining pattern of an independently generated antibody to the same protein.

Mass spectrometry

Mass spectrometry (MS) is a technique that measures the mass and charge of molecules. MS requires the transformation of samples to gaseous ions by an energy source such as a laser. The ions are then accelerated and subjected to a magnetic or electric field, and subsequently identified by a detector. The mass to charge ratio of the ionized molecules determine the time they take to reach the detector, by which they are detected and identified.

MS can be applied for protein analyses, with applications both in characterizing a single protein or analyzing complex biological samples. MS analysis can determine the identity and exact size of a protein with great accuracy, and can be used to identify the proteins and/or the presence of particular post-translational modifications in a complex sample. Typically, proteins are digested with proteolytic enzymes prior to analysis, in order to generate peptides that are sufficiently small to be analyzed by MS. The archetypal enzyme for this application is trypsin, which cleaves with high fidelity after arginine and lysine residues. On basis of a predicted cleavage pattern, detected peptides will then give the identity of the detected proteins.

In paper II of this thesis, an application of MS called LC-MS/MS was used to characterize the cleaved EphA2 receptor. LC-MS/MS involves separation of the sample by liquid chromatography and a two step MS-approach by which each ion/peptide species detected in the first MS round is then fragmented and analyzed by MS individually. This approach gives superior sensitivity, and allowed us to reach almost complete coverage of cleaved EphA2 protein. Since trypsin and FVIIa both cleave after arginine residues, we had to digest our sample with an additional enzyme in order to be able to discriminate peptides containing the FVIIa cleavage site from those cleaved by trypsin during sample preparation for MS. Chymotrypsin met these requiements, since it has non-overlappig cleavage spectrum compared to FVIIa, and cleaves sufficiently efficient to generate peptides that could be detected by MS.

N-terminal protein sequencing (Edman sequencing)

N-terminal sequencing, or Edman sequencing after the method's inventor the Swedish protein chemist Pehr Edman, is a way to sequence the N-terminal amino acid residues of a protein. First, the protein to be sequenced has to be isolated and purified. In the sequencing assay, the most N-terminal amino acid is chemically modified and then cleaved off the protein. It can then be extracted and is identified by chromatography. This cycle is then repeated to identify the next amino acid, so the number of sequencing cycles will determine how many amino acids that are sequenced.

In paper II of this thesis, N-terminal sequencing was used to identify the site in the EphA2 receptor where it is cleaved by TF/FVIIa.

Gene expression studies

Quantitative real-time PCR

qPCR assays are used for gene expression studies to determine the relative number of an mRNA transcript in a sample. Amplification and quantification is performed simultaneously as the probe binds to the amplified DNA and emits fluorescent light in each cycle, which is measured by a detector. A house keeping gene whose expression is assumed to be stable is used as an internal control, to which all results are normalized.

Manipulation of cellular gene and protein expression

siRNA mediated gene silencing

To study the consequences of loss of a particular gene or protein its expression can be inhibited by gene silencing. The cells own machinery for degradation of foreign RNA can be exploited for siRNA mediated gene silencing. siRNA duplexes, complementary to a stretch of sequence of the mRNA of the gene to be silenced is introduced to cells, most commonly by liposome-

based transfection. Once taken up by the cell, the siRNA duplexes are incorporated into the RISC complex, which associates with and cleaves the target RNA, thereby inhibiting gene expression. siRNA mediated gene silencing was used in papers I-III in this thesis, to study the effects of knockdown of the tyrosine kinase receptors IGF-1R, EphB2 and EphA2 on apoptosis and cell motility.

Transient overexpression of genes using plasmids

To facilitate its studies, a protein can be overexpressed in cells by introducing a plasmid containing the corresponding gene coupled to a strong and constitutively active promoter. Transient overexpression is then achieved, but by various techniques the gene construct can be introduced into cellular DNA, whereby a stable overexpression can be accomplished. In paper III of this thesis, TF was transiently ovexpressed from a plasmid containing the TF gene coupled to a CMV promoter.

In vitro studies of cellular functions

Apoptosis

A central event in programmed cell death is the activation of caspases through proteolytical cleavages. The presence of cleaved, i.e. activated, caspases can be studies as a marker of apoptosis, e.g. by Western blotting using cleavage specific antibodies, or by using fluorescently labeled probes to stain intact cells. The latter approach was used in paper I of this thesis, where cellular caspase activation was studied by using an automatic fluorescence microscope. In addition, changes in cellular morphology accompanying apoptosis such as nuclear alterations and reduction in cell size were recorded using this method.

Cell migration

Cell migration is commonly studied in biomedical research, by *in vitro* or *in vivo* assays. A commonly used in vitro assay is the *Transwell cell migration assay*, which was used in papers II and III of this thesis. The Transwell apparatus uses a special insert that divides a cell culture dish into two chambers, separated by a membrane with small pores. Cells are added to the upper chamber, and are allowed to migrate through the pores towards the chemoattractant in the lower chamber. The number of migrating cells can be quantified and provides a measure of the migrational ability of the cells and the strength of the chemoattractant.

Statistical analyses

In order to interpret scientific data collected from experiments, statistical analysis can be performed. Descriptive statistics describe and summarize the data, whereas statistical inference is used to estimate if the results indicate a true difference or are just examples of random variation. Statistical inference makes use of statistical tests, which indicate the probability that the null hypothesis is correct, i.e. that the results are due do random variation. This probability is expressed as a p value, which by convention is considered statistically significant if below 0.05 meaning that the null hypothesis will be true in one of twenty cases. Thus, in case of a p value below 0.05 the null hypothesis is rejected. In papers I-III of this thesis, Students t-test was used to compare experimental groups. In papers III-IV, characteristics of tumor specimens from a colorectal cancer cohort were compared by Chi-squared tests, and analyzed with respect to patient outcome by statistical survival analysis, using Cox regression and Log rank-tests, and visualized graphically by Kaplan-Meier curves.

Aims

-to explore the mechanisms behind the protective effect of FVIIa from extrinsic apoptosis (paper I)

-to use unbiased screening approaches investigate the involvement of RTKs in TF/FVIIa signaling (paper II)

-to further characterize cross talk between TF/FVIIa and novel RTK signaling partners on the cellular level using *in vitro* assays (paper II and III)

-to explore the significance of expression of TF and its signaling partners in human cancer material by immunohistochemistry (paper III and IV)

Results and discussion

Paper I

Background

The antiapoptotic effect of FVIIa was originally described in a paper published in 2003, where it was shown that FVIIa rescued cells from apoptosis induced by serum starvation[61]. Further work from our research group could demonstrate that FVIIa also had a protective effect against cell death induced by the death receptor ligand TNFα related apoptosis-inducing ligand (TRAIL), and that this occurred independently of both PAR1 and PAR2 activation[62]. The RTK Insulin-like growth factor I receptor (IGF-1R) has been implicated in antiapoptotic signaling in many contexts. The aim of this project was to further characterize the antiapoptotic signaling by TF/FVIIa and investigate a possible involvement of the IGF-1R.

Results and discussion

FVIIa antiapoptotic signaling was assayed by measuring caspase 3 and 8 activation, nuclear alterations and cellular morphology in MDA-MB-231 cells and PC3 cells treated with TRAIL and FVIIa using the Array Scan automated fluorescence microscope, where FVIIa partially reversed the effect of TRAIL.

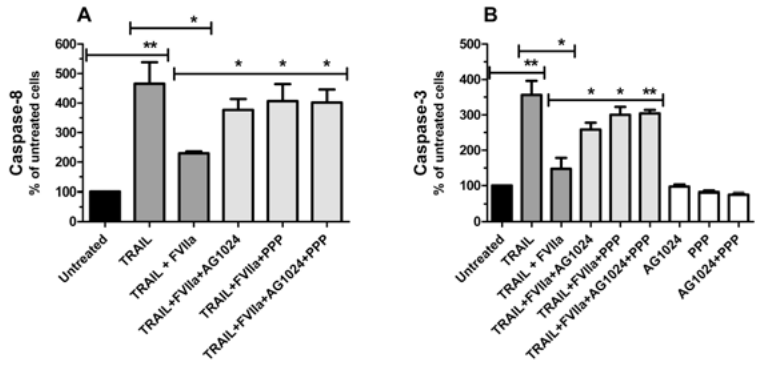

Figure 6. Antiapoptotic signaling by TF/FVIIa is reduced by IGF-1R-inhibitors. MDA-MB-231 cells were treated with TRAIL and FVIIa in the prescence or absence of IGF-1R inhibitors AG1024 and PPP and activation of caspases 8 (A) and 3 (B) were measured using the ArrayScan automated fluorescence microscope. Results are expressed as % of untreated control.

To evaluate the contribution of IGF-1R signaling to the antiapoptotic effect of FVIIa two synthetic IGF-1R inhibitors, AG1024 and picropodophyllin (PPP) were included, which abolished the protective effect of FVIIa (Fig. 6). These results were also confirmed by siRNA knock-down of IGF-1R in MDA-MB-231 cells. These results showed the requirement for IGF-1R in FVIIa antiapototic signaling, and to gain further insight into the mechanisms underlying these events we used the in situ PLA assay to investigate activation state and protein interactions of the IGF-1R after FVIIa treatment. FVIIa increased the interaction between TF and IGF-1R at the cell surface, and PLA using a pan-phospho tyrosine antibody showed an increased signal after FVIIa treatment indicating a transactivation of the IGF-1R by FVIIa. We also found that FVIIa increased the interaction between IGF-1R and the 14-3-3 family of proteins, previously implicated in IGF-1R antiapoptotic signaling[84] and providing a possible downstream signaling mechanism.

Furthermore, the IGF-1R was recently described to translocate to the nucleus to regulate gene expression[80]. By subcellular fractionation and in situ PLA we could demonstrate that FVIIa increased the nuclear trafficking and DNA binding of the IGF-1R to regulate expression of cyclinD1 mRNA, an IGF-1R target gene.

These data demonstrate the requirement for the IGF-1R in the antiapoptic signaling by TF/FVIIa. We show a direct activation by FVIIa, where the further signal transduction possibly involves 14-3-3 proteins that previously have been implicated in mediating survival signals from the IGF-1R. Longer term effects included translocation of the IGF-1R to the nucleus, the contributions of which to cell survival versus other effects of FVIIa will have to be elucidated.

Paper II

Background

When the work on this project was initiated, previous results from our group had demonstrated that TF/FVIIa sensitizes various cell types to PDGF-BB stimulated chemotaxis, and that this occurs through a transactivation of its receptor PDGFRβ, a tyrosine kinase receptor[57]. Although RTKs signaling is commonly implicated downstream of cell surface receptors of other classes, the only known RTK apart from PDGFRβ to be transactivated by TF/FVIIa by this time was the EGF receptor[109]. We therefore aimed to use screening technologies to investigate RTK involvement in TF/FVIIa signaling by an unbiased approach.

Results and discussion

To screen for RTK activation by TF/FVIIa we used commercially available antibody-based signaling arrays. Treatment with FVIIa, but not a PAR2 agonist peptide of MDA-MB-231 breast cancer cells and primary human fibroblasts resulted in a decreased phosphotyrosine signal for several members of the Eph receptor family. The array results implicated members of the Eph RTK family in FVIIa signaling, and we continued by investigating how FVIIa affected the expression of a selected number of Eph receptors. Western blots showed that FVIIa stimulation caused the appearance of reduced molecular weight species of the EphB2 and EphA2 receptors, starting at 0.5 nM FVIIa for EphB2 in all cell types tested.

Inhibitor experiments and enzymatic deglycosylation revealed that the reduced molecular weight isoforms were N-terminally truncated by an extracellular proteolytical cleavage, and we could also detect the released N-terminal EphB2 fragment in the cell culture supernatant. To delinate the cleavage mechanism we performed antibody blocking experiments and agonist stimulations and found that EphB2/EphA2 cleavage was dependent on TF, but occurred independently of PAR2 and downstream coagulation activity. The 10H10 anti-TF antibody which does not interfere with FVIIa binding but selectively blocks PAR2 activation[47] did not prevent EphB2/EphA2 cleavage in line with a PAR2-independent mechanism (Fig. 7).

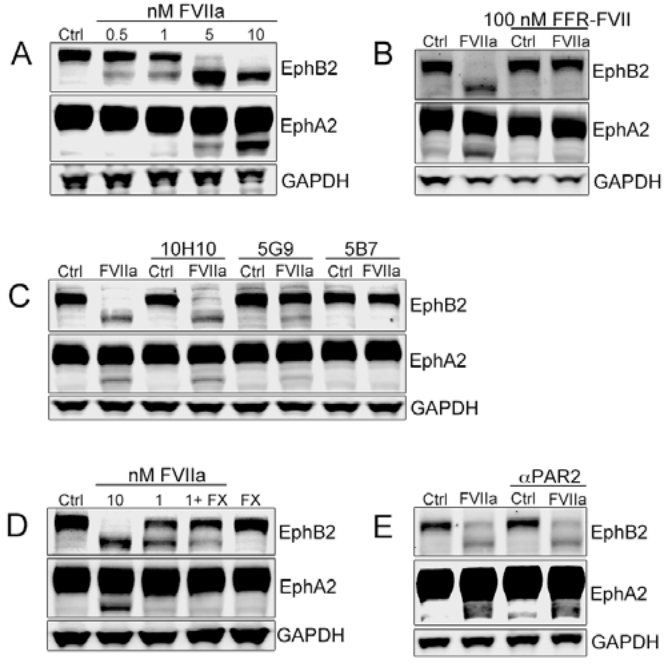

Figure 7. EphB2 and EphA2 are cleaved directly by TF/FVIIa. Images show Western blots of EphB2 and EphA2. GAPDH was used a loading control. All experiments were performed in MDA-MB-231 cells with 6 h of stimulation. (A) Concentration curves. (B) Cleavage is dependent on the proteolytical activity of FVIIa. FFR-FVII denotes active site inhibited FVII. (C) Cleavage is blocked by the 5G9 and 5B7 anti TF-antibodies, but not by the 10H10 anti-TF antibody. (D) Cleavage is independent of downstream coagulation activity. FX indicates 100 nM zymogen coagulation factor X. (E) Cleavage is not prevented by a PAR2-blocking antibody.

These results which ruled out the known mechanisms of TF/FVIIa signaling strongly suggested that EphB2 and EphA2 were cleaved directly by TF/FVIIa, which was supported by selective inhibition of the cleavage by serine protease inhibitors. We next used mass spectrometry and N-terminal Edman sequencing to identify the exact cleavage site, which was mapped to an arginine residue in the ligand binding domains of EphA2 and EphB2 (Fig. 8). In addition, the cleavage site was spanned by a conserved disulfide bridge. This disulfide appeared to be intact in a major fraction of the cellular EphB2/EphA2 pools, thus keeping the cleaved N-terminal fragment cell associated. Finally as a potential functional implication, using a Transwell chemotaxis assay we found that FVIIa potentiated cell repulsion by the EphB2 ligand ephrin-B1.

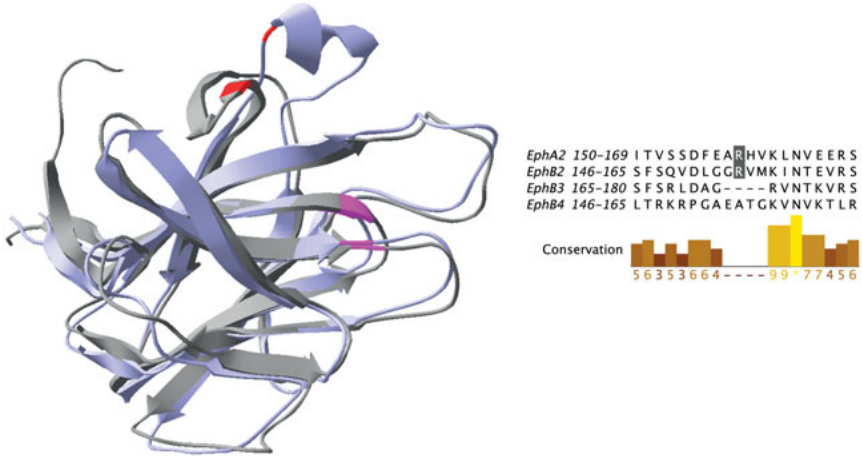

Figure 8. Identification of the cleavage site in EphA2 and EphB2. Left: 3D image of the arginine-159 (EphA2)/arginine-155 (EphB2) residue at the cleavage site (red) and the Cys70-Cys188 (EphA2)/Cys62-Cys184 (EphB2) disulfides (purple).EphA2 is shown in magenta and EphB2 in grey. Right. Alignment of the cleavage region in four Eph receptors. The arginines at the cleavage site are indicated in grey. The 3D image was generated using the Swiss Pdb viewer and the alignment using the Clustal Omega alignment tool and the Jalview software.

These results provide for the first time evidence of a proteolytic substrate of TF/FVIIa outside of the coagulation system other than PAR2. Importantly, the cleavage was found to occur after an arginine residue in line with the reported cleavage specificity of FVIIa, and the crystal strucures of EphB2 and EphA2 revealed that the cleavage site was readily accessible for a protease. We suggest that TF/FVIIa acts to potentiate ligand-dependent Eph-signaling, as demonstrated for the increase in ephrin-mediated cell repulsion by FVIIa.

Paper III

Background

The EphA2 receptor of the Eph RTK family is overexpressed in many types of cancers and has been proposed as an important oncogene[110]. In this project we aimed to extend our findings from paper II and further explore TF-EphA2 cross-talk in cancer.

Results and discussion

FVIIa stimulation caused a direct cleavage of EphA2 and a PI3 kinase dependent phosphorylation of serine-897 in the EphA2 cytoplasmic domain in MDA-MB-231 cells demonstrating that TF/FVIIa acts on EphA2 by two distinct mechanisms. While MDA-MB-231 cells express high amounts of

both TF and EphA2, EphA2 cleavage was also assayed in U251 cells with moderate TF expression. In this cell type forced overexpression of TF was needed for cleavage, demonstrating the requirement for high levels of TF for this proteolytical mechanism.

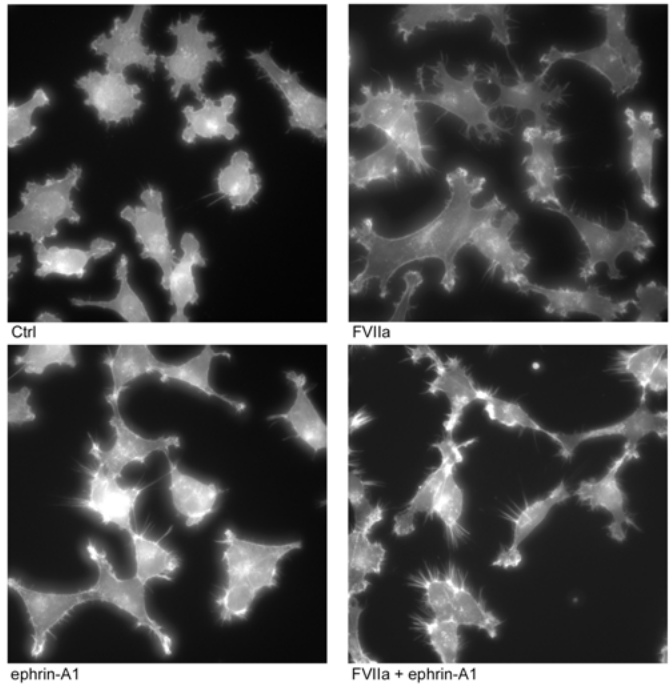

Figure 9. FVIIa potentiates cell rounding and retraction fiber formation by ephrin-A1. The images show epifluorescence micrographs of MDA-MB-231 cells stained by FITC-labelled phalloidin to visualize the actin cytoskeleton. Cells stimulated with both FVIIa and ephrin-A1 display an increased fraction of rounded cells with prominent retraction fibers.

The increase in serine-897 phosphorylation implied that FVIIa activated ligand-independent EphA2 signaling, the consequences of which was investigated by siRNA knock-down of EphA2. Using two different cell migration assays we found no changes in the response to FVIIa in EphA2-silenced cells, and instead we reasoned that FVIIa might regulate the way cells respond to the endogenous ephrin ligands, in line with our results from paper II.

EphA2 signaling is closely connected to the cytoskeleton, and to visualize cytoskeletal dynamics we stained cells with FITC-conjugated phalloidin which binds actin microfilaments. By confocal microscopy and in situ PLA we could show that TF and EphA2 co-localized extensively in MDA-MB-231 cells at sites with accumulation of polymerized actin fibers. When stimulated with the EphA2 ligand ephrin-A1, MDA-MB-231 cells transiently

became rounded and displayed retraction fibers as evidence of EphA2 activation targeting the cytoskeleton. Although not having any obvious effects on cellular morphology on its own, FVIIa significantly potentiated ephrin-A1-mediated cell rounding and retraction fiber formation, indicating a synergistic effect of FVIIa and ephrin-A1 on ligand-dependent EphA2-signaling (Fig. 9). This outcome required the protolytical activity of FVIIa and was independent of PAR2, in line with biochemical data showing a direct cleavage of EphA2 by TF/FVIIa. Inhibition of the RhoA effector ROCK, but not PI3 kinase, abolished cell rounding and retraction fiber formation by ephrin-A1 and FVIIa, in line with reports implicating RhoA signaling downstream of EphA2.

To demonstrate a possible clinical relevance of these findings we stained a colorectal cancer material for TF and EphA2 using IHC, and found that their expression patterns were significantly correlated. TF and EphA2 were foremost found in poorly differentiated tumors and metastases, and all but 2 cases positive for TF also expressed EphA2. These results demonstrate that the prerequisites for an interaction between TF and EphA2, i.e. co-expression in space and time, appears to be present in human cancer (Fig. 10).

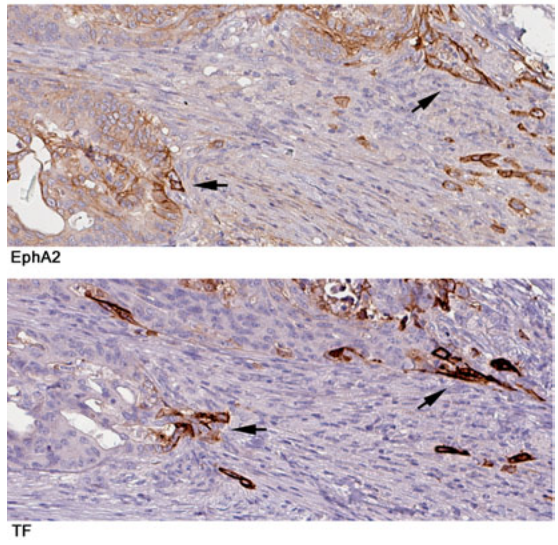

Figure 10. Micrographs showing IHC on serial sections of colorectal cancer tissue demonstrating expression of EphA2 (upper image) and TF (lower image) in tumor cells. Positive staining is visible as brown color.

Our data shows that TF/FVIIa might play a role in cancers in potentiating ligand-dependent EphA2 signaling. The role of EphA2 in malignancies is controversial, and ligand-dependent signaling has been considered tumor suppressive in some contexts. Importantly, several recent publications have

highlighted a tumor promoting role of ligand activated EphA2, e.g. in the development of a RhoA dependent amoeboid invasion style[102] and in how Eph-mediated repulsion may serve to increase cancer cell dissemination through dispersion from the main tumor mass[103]. Although the exact role of TF/FVIIa modulation of EphA2 signaling in these processes remains to be determined, our current results suggest this to be an interesting avenue for further studies.

Paper IV

Background

TF expression in the tumor cell compartment of various solid cancers is well described. However, TF expression in the stromal cells of human cancers has not yet been evaluated in larger clinical materials. We therefore aimed to investigate TF protein expression by IHC in all components of the tumor in two human colorectal cancer materials.

Results and discussion

Through collaboration with the Human Protein Atlas project, an antibody raised against human TF for use in the Protein Atlas project was identified. The antibody was subjected to the HPA validation pipeline, and displayed strong and specific staining for TF using IHC. Positive staining was observed in tissues previously reported rich in TF, such as the central nervous system, renal glomeruli, and certain epithelia such as the epidermis, cervix and bronchi. The HPA project has developed IHC on a panel of cell lines as a validation tool, where staining with the TF antibody correlated almost perfectly with relative RNA levels.

When applying the HPA TF antibody for IHC in human tissues, it labeled a thin layer of cells beneath the epithelium throughout the gastrointestinal canal, the so-called intestinal pericryptal sheath cell layer. To identify the origin of these TF positive cells we performed double stainings with markers for intestinal myofibroblasts (ACTA2), mesenchymal cells (vimentin) intestinal epithelium (CK20) and the epithelial basement membrane (collagen IV). The pericryptal sheath cell layer is generally believed to consist of myofibroblasts[111], but unexpectedly we found that TF and ACTA2 labeled to non-overlapping cell populations where the TF positive cells were located inside of the myofibroblast layer. The TF positive cells were located immediately adjacent to the basement membrane and were vimentin positive and CK20 negative, demonstrating a probable mesenchymal origin.

We next evaluated TF expression in two colorectal cancer cohorts using IHC on tissue microarrays and the same TF antibody. In addition to TF in tumor cells, we were interested in the fate of the TF positive pericryptal sheath cell population in cancers so both the tumor cell and stromal com-

partments were annotated during the pathological grading of the stainings. Tumor cell TF expression was present in fewer cases than expected, with around 25% of cases being positive in both cohorts and mostly poorly differentiated cases. Staining was frequently heterogenous, with small clusters of TF positive cells among otherwise negative specimens, and TF in tumor cells was not related to the clinical outcome. However, while the TF positive sheath cell layer surrounding the intestinal glands was intact in ademonas it was progressively lost in cancers and except for one case completely absent in metastases. Most positive cases displayed a heterogenous picture with positive and negative areas, while a significant fraction of cases was completely negative (Fig.11). In both cohorts, sheath cell TF was associated with low/intermediate tumor grade and CK20 expression, indicating that expression is lost in poorly differentiated cancers.

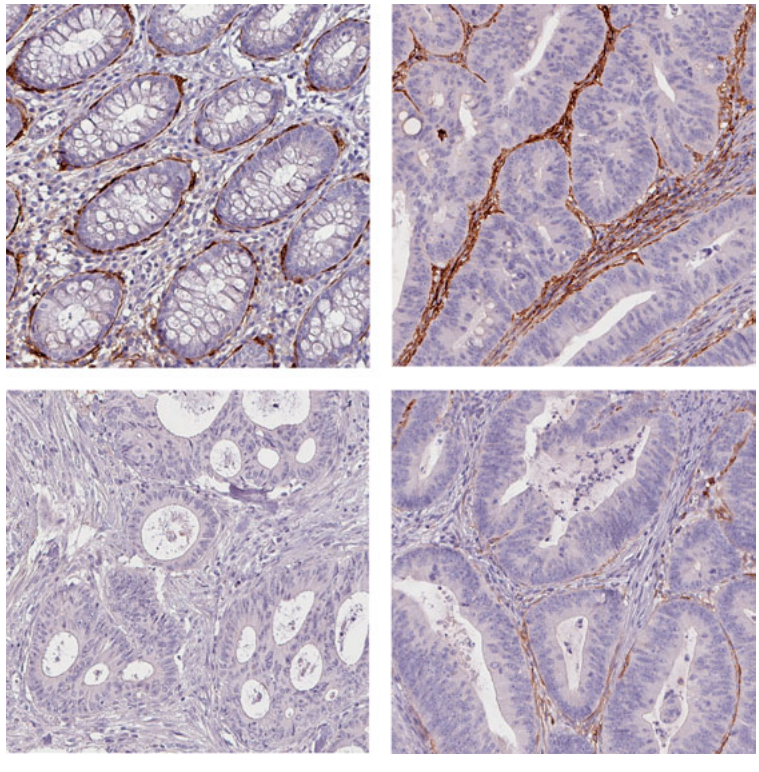

Figure 11. TF expression in the large intestine and colorectal cancer. Immunohistochemistry micrographs showing clockwise from upper row: normal colorectal mucosa, cancer specimen with high stromal TF expression, cancer with low stromal TF expression and cancer without detectable TF. TF is visible as brown staining.

In addition, we also stained a small series of larger tumor sections, which revealed that sheath cell TF was enriched in exophytic and central areas of the tumor. Interestingly expression was completely lost at the invasive front

of the tumor. In the CRC-BC cohort comprising around 300 cases sheath cell TF had a non-significant tendency to be associated with better survival. However, retained sheath cell TF expression was more common in rectal compared to colon cancers which was suggestive of a location specific role. Indeed, sheath cell TF was strongly associated with disease-specific and overall survival in rectal but not colon cancers (Fig. 12), and a prognostic indicator independent of disease stage, tumor grade and age.

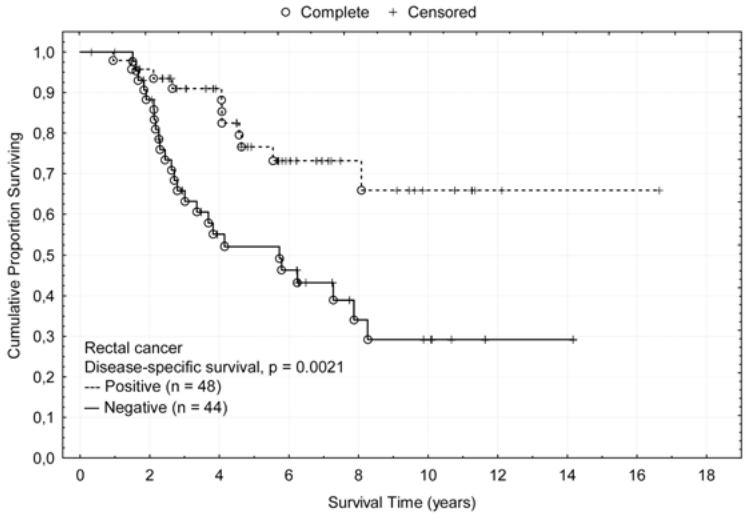

Figure 12. Sheath cell TF expression is associated with survival in rectal cancer. Kaplan-Meier curves showing survival of patients with tumors positive (dashed line) or negative (continuous line) for sheath cell TF.

These results demonstrate that TF has a dual expression pattern in colorectal cancer with sheath cell TF being present in earlier stages and with a possible role as a positive prognostic factor in rectal cancer, while TF expression in tumor cells occurs foremost in poorly differentiated cases.

General discussion and future perspectives

Novel signaling partners for the TF/FVIIa complex

In the first three papers, this thesis explored the involvement of tyrosine kinase receptors in TF/FVIIa signaling in an effort to understand the molecular events underlying the effects that coagulation activation exerts on cells and tissues. The prevailing view has been that proteolytic binary TF/FVIIa signaling occurs foremost through PAR2 cleavage. In this thesis we present evidence for additional signaling mechanisms, through cross-talk with

RTKs. In the first paper IGF-1R is shown to be necessary for antiapoptotic signaling by TF/FVIIa, which has previously been shown to be PAR2-independent. The initial proteolytic event in this signaling sequence was not identified and remains to be determined, but will likely be a cell surface protein implicated in IGF-1R signaling. In this context, we regard the IGF-1R as a signal transduction molecule rather than a receptor for an exogenous ligand, a role frequently taken by RTKs. Studies exploring protein interactions with TF or TF/FVIIa on the cell surface would be very interesting in this regard, in order to delineate the events that lead to IGF-1R activation by TF/FVIIa.

Papers II-III describe the discovery of EphB2 and EphA2 of the Eph family of RTKs as novel co-receptors and proteolytical substrates of TF/FVIIa. FVIIa has been thought to have rather narrow cleavage specificity, and only a few cleavage events are known. In this context, our results are of potential significance for the understanding of the biological effects of TF/FVIIa since they prove for the first time a direct cleavage of a non-coagulant protein other than PAR2. The notion of TF-Eph cross-talk is supported by the co-expression of EphA2 and TF in the same tumor specimens, although this is descriptive data and functional studies is required to establish a definitive role *in vivo* for this signaling mechanism. In any case, our results so far indicate that TF/FVIIa act to potentiate ligand-dependent Eph signaling, and suggest that this mechanism may be operative in cancer cells. One possibility is that the cleavage by a yet not fully characterized mechanism facilitates activation of ligand-dependent signaling. As described in the introduction of this thesis ligand-independent pre-clustering of Eph receptors also serves to enhance signaling, and additional role of TF/FVIIa might be to support such cluster formation. The large number of receptors and ligands confers significant complexity to the Eph system and complicates the study of a single Eph. Further studies will likely have to de conducted using a purified system, e.g. a cell with overexpression of one single Eph to be studied.

Cleavages in Eph receptors have in other cases been associated with enhanced receptor activation and downstream signaling[112,113] which lead us to speculate that cleaved EphB2/EphA2 somehow gain an ability to increase ligand-dependent signaling. We found that the cleavage site was spanned by a disulfide bond, suggesting that its reduction is necessary for dissociation of the N-terminal fragment. The fact that the disulfide bond appeared to be intact in a major fraction of the cellular EphB2 and EphA2 pool may explain why cells still responded to ephrin ligands. As disulfide reduction is emerging as an important mechanism in regulation of extracellular proteins[2] it would be of considerable interest to examine this further, for example by investigating the possible role of oxidoreductases such as protein disulfide isomerase in TF-Eph cross-talk. Another interesting option would be to determine the redox status of the EphA2/EphB2 disulfide by mass spectrometry and find out whether it could be reduced by stimuli such as ligand stimu-

lation. Since we found the cleaved EphB2 fragment in free form in the cell culture supernatant it appears that it is indeed released from cells. According to the crystal structures the disulfide is surface located and accessible to solvent, which implies that it may be cleaved by reductase. Thus there are several indications that this might be an allosteric disulfide bond, which will have to be confirmed by experimental data.

EphB2 was consequently cleaved by lower concentrations of FVIIa than EphA2, and endogenous TF was sufficient to support cleavage in fibroblasts and U251 cells, while high TF expression was required for detectable EphA2 cleavage. As subnanomolar FVIIa concentration cleaved EphB2 in all cell types, this may be a mechanism of importance also in non-malignant cells. Monocytes express EphB2 upon activation[114], and interact with ephrins expressed on the vascular endothelium. Our results suggest that plasma FVII concentrations should be more that sufficient to generate EphB2 cleavage in TF expressing monocytes, which merits further investigation of the TF-EphB2-ephrin-B axis in this cell type during inflammation. An ongoing debate in the TF research field concerns the role of the cellular pools of cryptic and active TF in coagulation and signaling. We found that EphB2 cleavage was saturated at concentrations at or below the range of K_D values that have been reported for the TF-FVIIa interaction, and below concentrations where TF binding of FVIIa is saturated. This dissociation may mean that FVIIa bound to active TF foremost cleaves EphB2. As we found that formation of the TF/FVIIa/FXa complex did not support EphB2 cleavage our results suggest an additional non-coagulant role for active TF.

In paper II we examined 4 Eph receptors and found that two of these were cleaved in response to FVIIa. As mentioned above the Eph family contains 14 members, and as the arginine at the cleavage site is conserved in many of these additional Ephs they might also be cleaved by TF/FVIIa. As for all protelytical events, co-localization in space and time is required but interactions between TF and other Ephs will likely be found.

TF expression in the large intestine and colorectal cancer

The last paper in this thesis had a different focus than the first three and was initiated with the aim to study expression of TF and related proteins in colorectal tumors by IHC. As IHC is crucially dependent on antibody quality we put great effort into validating all antibodies, by using the HPA validation pipeline. We identified a TF antibody made as a part of the HPA effort, which met all validation requirements and with a high likelihood was specific for TF, which was subsequently used to stain two colorectal cancer materials. A number of studies have described widespread TF expression in colorectal cancer as well as in solid tumors in general. We found lower rates of TF positivity than expected, with around 25% of cases being positive for TF in both cohorts. Nor did we find any relationships between disease stage or

survival and tumor cell TF expression. The possible explanations for these discrepancies include the use of tissue microarrays were the number of positive cases could be underestimated. Another possibility that has to be taken into account is that previous studies overestimated TF expressing due to non-specific antibody reactivity, which is hard to judge since many of these publications did not include antibody validation data.

However, when examining normal colorectal mucosa we found that TF expression in this case was confined to a thin cell layer lining the intestinal glands, which were subsequently identified as a vimentin-positive and ACTA2-negative cell population likely representing the innermost layer of the intestinal pericryptal sheath cells. Using two different cohorts we could further show that this source of TF was progressively lost after the adenoma-to-carcinoma transition and was almost completely lost in metastases.

Compared to other epithelial tissues in the human body, this particular expression pattern with strongly TF positive cells lining a mostly negative epithelium was exclusively found in the gastrointestinal canal. An obvious function for TF at this location is to prevent gastrointestinal bleedings, in accordance with the hemostatic envelope concept. The gastrointestinal mucosa provides the first-line defense against various pathogens entering by this route, and TF and the coagulation system may equally well support this function in line with its role in innate immunity. However, this does not answer why TF expression is confined to subeptihelial cells, compared to squamous epithelia which are rich in TF. Pericryptal sheath cells are thought to function in maintenance of the gastrointestinal epithelium and one could speculate in a role for TF-mediated signaling in this regard.

Since this study was descriptive, the definitive function of TF positive sheath cells during tumorigenesis remains to be determined, but we argue that our results are strongly suggestive of tumor suppressive role at least in later stages. This is supported by the increasing loss of sheath cell TF along the progression of a cancer. As shown by intact tumor sections, the loss of sheath cell TF at the invasive front indicates that it needs to be downregulated in order to allow for tumor invasion. A related question that remains to be answered is whether TF is just marker of intestinal sheath cells, or actively plays a tumor suppressive role in this context, and analogously, whether this sheath cell population is lost entirely or merely downregulates TF. We observed abundant ACTA2 expression in the stroma of colorectal cancer, which in this context also identifies cancer-associated fibroblasts (CAFs), a cell type with characteristics of both fibroblasts and myofibroblasts found in cancer stroma. One source of CAFs is resident fibroblasts, which are differentiated into CAFs by factors released from tumor cells[115]. One possibility may be that TF expression in sheath cells is lost during this process under influence of stimuli from tumor cells.

Another intriguing finding of this study was that retained sheath cell TF expression was more common in rectal cancer and a prognostic factor in

rectal cancer only. Rectal and colon cancers display both biological differences and distinctions in clinical management and this may reflect the heterogeneity of colorectal cancer as a whole.

In summary, this thesis presents new insights into the molecular events that follow upon formation of the TF/FVIIa complex at the cell surface. We describe that TF/FVIIa directly cleaves a novel population of cell surface receptors, which opens up exciting new avenues for further research. In addition, we show that TF is a marker for a specific cell population in the gastronintestinal mucosa, and might therefore function as a prognostic biomarker in rectal cancer. Whether TF expressed by these cells plays a mechanistic role in cancers developing in the large intestine remains to be determined, and will be another important aim for further studies. As new techniques and research tools are emering in medical research, so are the possibilites to delineate the physiological role of the findings of this thesis in more detail. Using e.g. genome editing techniques to modify the expression or function of TF and Eph receptors in a single cell type or compartment in an *in vivo* model system would open up possibilities for mechanistic studies that would not have been possible previously. The ultimate goal of such studies would be to identify opportunities for applications in health care and medicine, where findings could be developed into drugs or diagnostic tests for medical use in patients with conditions involving a deregulation of the coagulation system.

Conclusions

- The antiapoptotic effect of FVIIa is independent of PAR2 but requires signaling by the IGF-1 receptor
- FVIIa causes IGF-1R nuclear translocation and DNA binding
- The RTKs EphB2 and EphA2 are cleaved directly by the TF/FVIIa complex after a conserved arginine residue and independently of PAR2 activation and downstream coagulation proteases
- FVIIa increases ephrin-B1-induced cell repulsion mediated by EphB2
- TF and EphA2 co-localize at cell-cell contacts and at sites with accumulation of polymerized actin cytoskeleton
- FVIIa potentiates cell rounding and retraction fiber formation by the EphA2-ligand ephrin-A1.
- TF is a marker of the innermost layer of pericryptal sheath cells in the large intestine
- TF is expressed in both the stromal and tumor cell compartments in colorectal cancer
- TF expression in pericryptal sheath cells in colorectal cancer is lost in poorly differentiated tumors and is a prognostic marker in rectal cancer independent of disease stage, histological grade and age.

Populärvetenskaplig sammanfattning på svenska

Koagulationssystemet har som uppgift att förhindra att vi blöder ihjäl efter en skada genom att bilda ett koagel i skadeområdet och således motverka fortsatt blodförlust. Samtidigt är det av yttersta vikt att blodet hållas flytande inne i blodkärlen så att vi inte drabbas av blodproppar, och för att balansera de här två funktionerna har ett komplext system utvecklats. Koagulationssystemet innefattar därvid en rad komponenter i samspel. Bland dessa kan nämnas trombocyter, vilka är små celler som binder till skadade kärl och bidrar till koagelbildningen, och koagulationsfaktorer, vilka är i blodet cirkulerande proteiner som vid skada aktiverar varandra och gör att ett nätverk av proteinet fibrin fälls ut och stabiliserar koaglet.

Trots väl utvecklade system för att hålla koagulationssystemet i balans är denna ofta rubbad i en rad sjukdomstillstånd, med ökad benägenhet för blodpropp eller blödning som följd. Man har även upptäckt att koagulation och inflammation är tätt sammankopplade system i kroppens försvar mot skador och patogener. Koagulationsaktivering leder bl.a. till ett starkare inflammatoriskt svar, och koagulationsfaktorer kan direkt påverka celler genom att binda till ytreceptorer och aktivera cellsignalering.

Tissue Factor, förkortat TF, eller på svenska vävnadsfaktorn är ett membranprotein som bildar ett komplex tillsammans med sin ligand koagulationsfaktor VII/VIIa och fungerar som startpunkten i koagulationsprocessen. Som andra koagulationskomponenter kan TF/FVIIa-komplexet även direkt påverka celler genom att aktivera cellsignalering via klyvning av andra proteiner och receptorer.

I de första delarbetena i den här avhandlingen har vi undersökt de molekylära mekanismerna vid cellsignalering som initieras av TF/FVIIa-komplexet. Sedan tidigare är det känt, bl.a. utifrån resultat från vår forskargrupp, att TF/FVIIa skyddar cancerceller från programmerad celldöd, s.k. apoptos. I arbete I visar vi att detta sker i samspel med en annan cellytereceptor, som benämns IGF-1-receptorn (IGF-1R). Genom att på olika sätt hämma IGF-1R-signalering upphör FVIIa:s skyddande effect mot celldöd, och vi visar även en direkt interaktion mellan TF och IGF-1R på cellytan.
Även om IGF-1R är en cellytereceptor med uppgift att binda extracellulära ligander och föra en signal in i cellen, har det visat sig att cellytereceptorer även kan utföra andra funktioner i cellen. I överensstämmelse med sådana

fynd kan vi även visa att stimulering med FVIIa leder till att IGF-1R translokerar till cellkärnan och direkt binder DNA, för att där slå på genuttryck.

I delarbete II upptäckte vi att TF/FVIIa direkt kan klyva EphB2 och EphA2, två medlemmar i en grupp cellytereceptorer som kallas Eph-receptorer vilka har en viktig roll i kommunikation mellan celler. Genom att använda en panel av hämmare och blockerande antikroppar kunde vi visa att EphB2 och EphA2 klyvs direkt av TF/FVIIa och att detta sker oberoende av de hittills kända signaleringsvägarna för TF. Proteaser, dvs. enzym som klyver andra proteiner, har olika klyvningspreferenser och klyver bara proteiner vid bestämda aminosyresekvenser. Genom att använda proteinkemiska tekniker som masspektrometri och N-terminal Edmansekvensering identifierade vi det exakta klyvningsstället i EphA2 och EphB2 till en Arginin-aminosyra i den ligandbindande delen av receptorerna. För FVIIa vet man sedan tidigare att den bara klyver efter aminosyran Arginin, varför de här resultaten stämmer överens med en direkt klyvning av TF/FVIIa. Även om de flesta proteaser man känner till har ett antal substrat har tidigare endast ett fåtal sådana varit kända för TF/FVIIa, varför de här resultaten bidrar till förståelsen för hur koagulationssystemet och TF/FVIIa påverkar celler och vävnader.

I delarbete III fortsatte vi att undersöka samspelet mellan TF/FVIIa och EphA2-receptorn. Då EphA2 i många andra studier beskrivits som ett protein viktigt för cancercellers malignitet och spridning använde vi två cancercellinjer som modellsystem. Med konfokalmikroskopi kunde vi demonstrera att EphA2 och TF uttrycks i samma subcellulära strukturer, med en ackumulering i cellmembranet och vid kontakter mellan celler, vilket är en förutsättning för en direkt klyvning. EphA2-signalering har i tidigare arbeten ofta kopplats till cellrörlighet via påverkan på cellskelettet, och vi kunde i det här arbetet visa att stimulering med FVIIa förutom en direkt klyvning av EphA2 även gjorde att det cellulära svaret på ligandstimulering av EphA2 potentierades, med ändrad cellmorfologi och omorganisering av cellskelettet som resultat. Slutligen kunde vi även visa att TF och EphA2 finns uttryckta tillsammans i kolorektalcancerprover från människa, vilket visar att förutsättningarna finns för ett samspel på molekylär nivå mellan TF och EphA2 i cancer.

I det fjärde delarbetet beskriver vi framtagandet och valideringen av en ny antikropp mot TF, som vi sedan använder för studier av TF-uttryck med immunohistokemi i normal tarmvävnad och kolorektalcancer. I normal tjocktarmslemhinna är uttrycket av TF koncentrerat till en specifik population av stödjceller lokaliserade precis under tarmepitelet, ett uttryck som förändras och försvinner i kolorektalcancer. Hos patienter med rektalcancer är överlevnaden även signifikant försämrad hos patienter där TF-uttrycket har försvunnit, och vi föreslår därför TF-utryck i tumörstroma som en möjlig prognostisk biomarkör för rektalcancer.

Sammanfattningsvis har vi genom de i den här avhandlingen ingående studierna bidragit med ny kunskap kring de molekylära processer som sker när TF/FVIIa-komplexet bildas på ytan av TF-uttryckande celler, och kring hur TF/FVIIa samspelar med andra cellytereceptorer för att påverka celler och vävnander. Vidare har vi visat hur TF är en markör för specifik stödje-cellspopulation i tarmslemhinnan, och därmed skulle kunna fungera som en prognostisk biomarkör i rektalcancer.

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