Chapter 1 - General Introduction and Outline of the thesis

The coagulation cascade

The coagulation system is a vital mechanism that helps to prevent blood loss. Coagulation is the result of a complex cascade of enzymes that was first proposed in 1905 by Morawitz [1]. The coagulation cascade is divided in two distinct parts; the extrinsic pathway which plays a major role in the initiation phase, and the intrinsic pathway which is responsible for amplification and propagation of clotting. The initiator of the extrinsic pathway is Tissue Factor (TF), a membrane tethered protein mainly expressed by the cells that reside in the medial and adventitial layer of the vessel wall (i.e. vascular smooth muscle cells and fibroblasts). TF is normally not expressed on endothelial cells as direct contact with circulating coagulation factors may cause excessive clotting. However, stimulation with inflammatory mediators results in the expression of TF on endothelial cells and may be responsible for thrombotic complications [2-5].

Coagulation is initiated when vascular integrity is compromised, such as after wounding. As a consequence the endothelium is damaged and TF is exposed to the blood. The blood-borne zymogen FVII, which is produced in the liver, forms a high-affinity complex with blood-exposed TF upon which FVII is converted to the enzymatically active protease FVIIa. The TF:FVIIa complex then activates coagulation factor X (FX) which leads to the formation of trace amounts of thrombin. Thrombin amplifies its own production through activation of a feedback-loop consisting of subsequent activation of factor XI (FXIa), the tenase complex (protease FIXa in complex with its co-factor VIIIa (FVIIIa)) and the prothrombinase complex (protease FXa in complex with its co-factor factor Va (FVa)). As a result, fibrinogen will be converted to fibrin and a clot will form. This feedback loop takes place on the surface of activated platelets that provide the negatively charged phospholipid layer essential for coagulation factor activation

Blood coagulation is subject to regulation by coagulation inhibitors. Coagulation initiation is regulated by TFPI which FXa-dependently inhibits TF:FVIIa function. In addition, coagulation propagation is effectively regulated by antithrombin that targets FIXa, FXa and thrombin [6]. The Protein C/Protein S pathway is another negative regulator of the coagulation cascade. As the levels of thrombin increase, thrombin binds to thrombomodulin on endothelial cells. This results in thrombin-dependent cleavage of Endothelial Protein C Receptor (EPCR)-bound Protein C. Generated Activated Protein C (APC) in complex with protein S can proteolytically inactivate FVIIIa and FVa to terminate blood clotting [7] **(Fig 1)**.

The complex nature of the coagulation cascade assures tight regulation of this process in order to prevent excessive or inadequate clotting. Nevertheless, aberrant expression or

activation of coagulation zymogens and cofactors may cause clotting and bleeding problems. For instance, deficiency in FVIII or FIX cause bleeding problems in hemophilia A and hemophilia B patients respectively. In contrast, excessive activation of coagulation factors, or defects causing prolonged activity (e.g. Factor V^{Leiden} mutation, yielding a FV variant that is resistant to APC) may result in a hypercoagulable state, and thrombus formation in veins [7, 8].

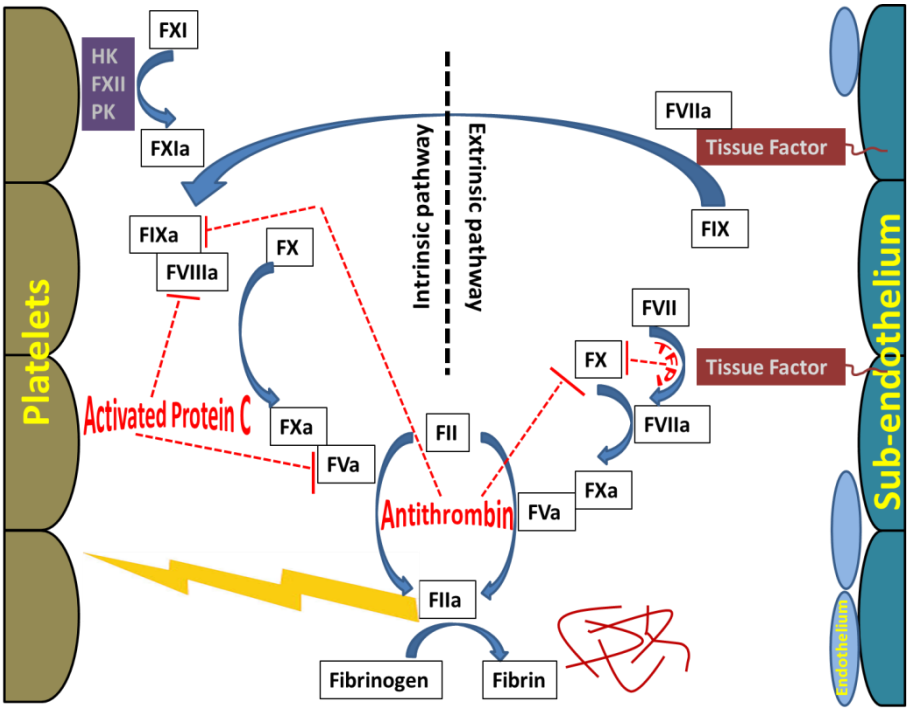

Fig.1 The coagulation cascade Sub-endothelial cells exposing TF, and platelets facilitate activation of a complex pathway regulating coagulation. Blue arrows represent activation and red lines indicate inactivation of the coagulation factors.

Coagulation factor signaling

Other than regulating blood clotting, coagulation factors may also signal via G-protein coupled Protease Activated Receptors (PARs) on cells. PARs are seven-span transmembrane proteins belonging to a family consisting of four members: PAR1-4. Upon activation, their N-terminus is cleaved and the newly formed N-terminus binds to the second extracellular loop of the receptor thus initiating downstream signaling. PARs are targeted by specific proteases. PAR1, PAR3 and PAR4 are typically cleaved by thrombin [9]. However, PAR1 can also be activated by plasmin [10], APC [11], FXa [12] and matrix

metalloproteases [13]; PAR2 is the only PAR that cannot be activated by thrombin but it is responsive to trypsin [14], mast cell tryptase [15], membrane-type serine protease 1 [16], bacterial proteases [17], TF/FVIIa [18], TF/FVIIa/FXa [19] and FXa-dependent cleavage [18].

Platelet activation and aggregation is a crucial step in coagulation. Platelet activation by thrombin is mainly orchestrated by PAR1/PAR4 on human platelets [20, 21]. PAR1 regulates platelet activation at relatively low levels of thrombin while PAR4 cleavage results in platelet activation when thrombin concentration is high. Most likely, PAR1 and PAR4 act in concert on human platelets [21]. On mouse platelets, thrombin-dependent activation occurs through PAR3/PAR4 targeting [22]. PAR3 does not appear to be directly activated by thrombin but to act as a coreceptor for PAR4 by optimally positioning thrombin on the platelet surface towards PAR4 [23].

All four PAR members are expressed on endothelial cells which allows for a broad spectrum of actions regulated by thrombin [24, 25]. PAR1 activation by thrombin leads to vWF secretion from Weibel Palade bodies in order to amplify coagulation by recruiting platelets to the wound area and acting as a carrier for FVIII [26]. Additionally, to avoid uncontrolled trafficking of biological substances, endothelial cells form a structural barrier that is sealed with tight junctions [27]. PAR1/thrombin activity increases the permeability of endothelial cells which results in an influx of coagulation factors to the tunica media. Overall, the relocalization of these factors across the endothelium may lead to more efficient coagulation, but potentially also to pro-thrombotic complications [28]. Apart from coagulation, thrombin/PAR signaling is also involved in inflammatory responses. Thrombin stimulation of endothelial cells increases cell surface P-selectin levels to promote leukocyte binding [29, 30] and triggers the release of cytokines [31].

Apart from PARs, endothelial cells express/expose a unique set of proteins modulating the anti-coagulant Protein C pathway. APC activates PAR1 in an EPCR-dependent manner [11, 32]. Interestingly, APC-induced PAR1 activation and thrombin-induced PAR1 activation result in opposing cellular effects: APC-driven PAR1 cleavage triggers anti-apoptotic and anti-inflammatory signaling [32, 33], and decreases endothelial permeability [34] while thrombin-directed PAR1 activation increases endothelial permeability [28] and is involved in inflammatory responses [31]. These differences are most likely dictated by the strength of the downstream signal. Thrombin/PAR1 results in a relatively strong activation of the $G_{12/13}$ and Rho pathways, to disrupt the barrier function [35] whereas a weak signal resulting from APC-induced PAR1 signaling triggers Sphk1 and S1P release resulting in the opposite [36].

Tissue Factor signaling

Apart from being the initiator of coagulation cascade, TF supports cellular signaling. FVIIa in complex with TF activates PAR2 [18]. FVIIa-dependent PAR2 signaling is further supported by integrins. Antibodies either inhibiting β1 integrins or disrupting β1/TF complexes have a negative effect on PAR2 signaling [37]. Upon PAR2 activation, PKCα localizes to the cell membrane via the phosphatidlycholine-dependent phospholipase C pathway, and phosphorylates the TF cytoplasmic tail (TF-ct) on Ser 253 followed by p38α-dependent Ser 258 phosphorylation [38, 39]. TF's extracellular domain is involved in the regulation of coagulant activity and cell survival by the phosphorylation of STAT5 [40]. In addition, the FVIIa:TF complex regulates the transcription of wound repair response genes (i.e IL-1β, egr-1, hbEGF) in keratinocytes [41]. TF's cytoplasmic domain does not play a role in PAR-dependent p42/44 activation [42]. In contrast, activation of p38 requires the presence of TF-ct [43] and the same holds true for adhesion, migration and spreading of fibroblasts [44]. In an angiogenic context, the TF-ct inhibits PAR2-dependent angiogenesis while its phosphorylation reverses this inhibition [45]. Similarly, the TF-ct blocks α3β1 integrin function and consequently cell migration , but upon PAR2 -dependent TF-ct phosphorylation this inhibition is released [46]. Overall, these data suggest that TF drives cellular events in TF-ct-dependent manners (e.g. migration and angiogenesis), and in TF-ct-independent manners (e.g. gene expression and coagulation).

In addition to physiological settings, TF-dependent signaling also plays a role in pathophysiological (i.e. cancer) setting. In breast cancer, disease progression is influenced by the TF/FVIIa/PAR2 axis. Deletion of PAR2, but not PAR1 in a spontaneous model of breast cancer resulted in reduced tumor size, metastasis and angiogenesis [47]. Additionally, injection of MDA-MB-231 mfp cells into the mice in the presence of PAR2-blocking antibodies resulted in smaller tumors whereas PAR1 antibodies were without effect [37]. The same proliferative and angiogenic effect of TF/FVIIa/PAR2 was observed in melanoma models [48]. TF/FVIIa/PAR2 signaling increases the expression of pro-angiogenic factors (i.e. VEGF, Cxcl-1, Cyr61, IL-8) [49-51] and there is a correlation between TF expression and microvessel density in lung [52], gastrointestinal [53], prostate cancer [54] and gliomas [55]. FVIIa also increases uPAR levels in pancreatic cancer cells [56, 57] and ovarian cancer cells [58] which is believed to regulate tissue reorganization and metastasis [56, 57]. In support, FVIIa signaling was shown to directly regulate melanoma metastasis [59]. TF/FVIIa also upregulates BcL2 in cancer cells to inhibit apoptosis [60].

TF may also signal and regulate cellular processes independent of PARs. In endothelial cells, TF in complex with Akt increases CCL2 levels that attract smooth muscle cells to form

mature vessels [61, 62]. Moreover, downregulation of TF results in diminished angiogenic potential and stimulation with PAR2 agonist rescued the angiogenic phenotype only partially which suggests the involvement of PAR2-independent pathways in TF-driven angiogenesis [62]. Incubation of endothelial cells only with exogenous TF activates the JNK-SAPK pathway and increases proliferative Elk-1 levels [63]. The increasing numbers of endothelial cells may allow the incorporation of those cells into the newly formed vessels in order to support angiogenesis. Moreover, TF in complex witha proteolytically inactive form of FVIIa (FVIIai) activates CREB and NFκB which are both involved in cell survival [63]

Tissue Factor decryption

Cellular levels of TF antigen do usually not correlate with cellular pro-coagulant activity, as most of the TF pool resides in a non-coagulant/cryptic form on the cell membrane [64]. So far, the exact mechanism regulating the activation of TF remains controversial. Several theories were suggested to explain the decryption process: phosphatidylserine exposure, localization of TF in lipid rafts , TF dimerization and disulfide switch model.

TF encryption/decryption was originally thought to be dependent on phosphatidylserine exposure. In a resting cell, phosphatidylserine (PS) is kept in the inner leaflet of the lipid bilayer by flippases whereas floppases transport phosphatidylcholine (PC) to the outer leaflet, thus creating an asymmetrical cell membrane. Stimulation of cells with Ca^{2+} ionophore or apoptotoic signals block the action of flippase and enable scramblase activity which in turn transports PS and PC across the lipid bilayer in an unselective manner [65]. Due to increased PS levels on the outer leaflet, a negatively charged surface is formed which allows the optimal complex formation between coagulation factors [66].

Alternatively, it has also been suggested that TF's positioning in different membrane compartments might be involved in decryption. Lipid rafts are rigid microdomains that contain elevated levels of cholesterol and glycosphingolipids [67]. Some researchers argue that rafts provide the optimal environment for TF to perform its coagulant function whereas others claim that these domains are unsuitable for efficient coagulation [68-70]. Obviously, further research is required to prove or refute this theory.

A third model argues that TF homodimers are cryptic and TF monomers are the coagulant pool [71]. Stimulation of HL60 cells with TF activity inducing reagent, calcium ionophore [72], leads to a decrease in TF cross-linking [71]. Conversely, the fusion of a leucine zipper motif to soluble TF not only promotes TF dimerization but also does not affect TF's amidolytic or Xa generation activity [73].On the basis of the aforementioned observations, it is clear that more studies should be performed to clarify the dimerization theory.

The last theory to explain TF encryption/decryption is the disulfide switch model. Both human TF (hTF) and mouse TF (mTF) contain an allosteric cysteine-cysteine bond in their extracellular domain (Cys^{186}-Cys^{209}, Cys^{190}-Cys^{213} respectively) which is subject to regulation by protein disulfide isomerase (PDI). Reduction of these disulfide bonds results in the formation of cryptic or signaling TF whereas oxidation causes an increase in TF-dependent coagulation [74, 75]. TF contains an additional extracellular disulfide bond between cysteines at the positions 49 and 57. Although this disulfide bond but does not appear to have an influence on TF's coagulant function, it was suggested that this disulfide bond may be involved in the regulation of redox activity at the C186-209 positions [76, 77]. Finally, the TF allosteric disulfide bond appears to be regulated by nitric oxide and glutathione, as both agents are capable of disrupting the disulfide bond and TF activity [74, 78].

There are also studies supporting the notion that Cys^{186}-Cys^{209} disulfide bond is not essential for the regulation of TF's pro-coagulant activity [79]. It is important to note that in these studies in order to mimic the reduced form of TF, cysteines were exchanged into alanine or serine residues which cause a misfolding problem in TF. As a result, the mutants have lower surface antigen levels than wild type TF. Although these studies attempted to express coagulant activity per TF molecule, excessive membrane surface might have caused an overestimation of the coagulant function for the mutants.

Alternatively Spliced Tissue Factor

A decade ago, another TF isoform, alternatively spliced Tissue Factor (asTF), was discovered [80]. The splicing machinery may choose between the inclusion or exclusion of exon 5 of the TF gene to form TF (nowadays often referred to as full length TF; flTF, to avoid confusion) or asTF respectively. Exclusion of exon 5 also causes a frameshift in the TF mRNA sequence. Consequently, human asTF (hasTF) contains a 40 amino acid unique C-terminus, while the first 166 amino acids in human flTF (hflTF) and hasTF are identical [81]. In addition, asTF lacks the transmembrane domain and is a soluble protein. The expression of asTF is tissue and cell type specific. HasTF is expressed by promyelocytic leukemia cells (HL-60), CD14+ monocytes, TNF-α and IL-6 treated endothelial cells, smooth muscle cells, gastric carcinoma (KATO III, SNU-5 and MKN-74), colon carcinoma (HCT 116), neuronal glioblastoma (U343), squamous cell carcinoma (A431), melanoma (WM1341B, WM983A), pancreatic carcinoma (Capan-2) cell lines, and is also expressed in pancreas, placenta, lung and human thrombi [80, 82-84]. hasTF also has a mouse homologue (masTF) with a slightly longer C-tail [85] that is localized in kidney, lung, stomach and murine thrombi [85, 86].

Exon 5 of the TF pre-mRNA contains exonic splicing enhancer (ESE) sequences that interact with a set of splicing factors (i.e. SR proteins) to regulate inclusion or exclusion of exon 5 **(Fig.2)**.

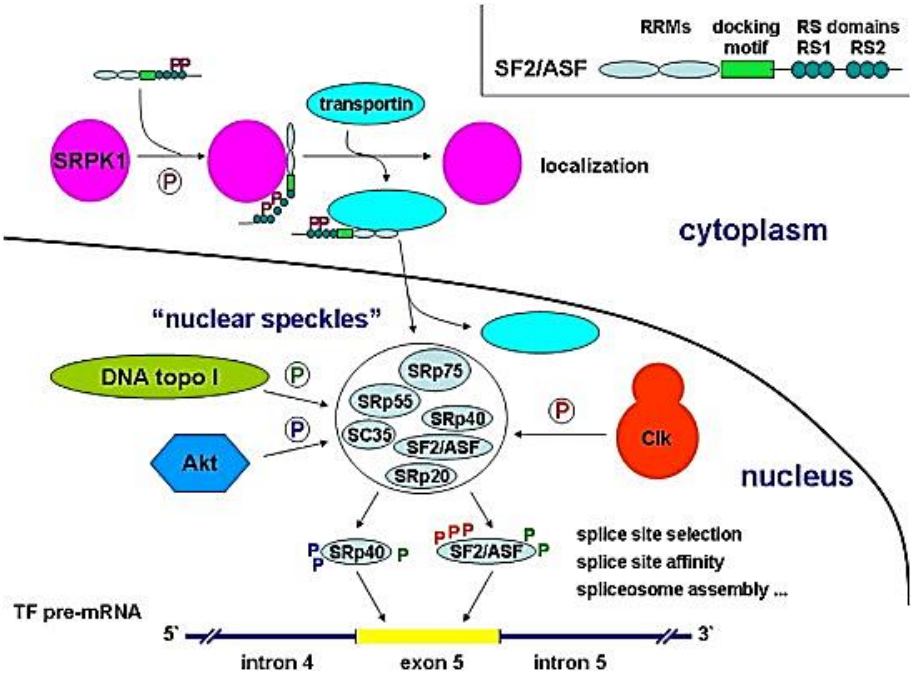

Fig.2 Regulation of TF pre-mRNA splicing by splicing regulator proteins. SR protein resides in the cytoplasm and are transported into the nucleus following phosphorylation by SRPK1. SR proteins are then additionally phosphorylated by a variety of SR protein kinases to regulate their interaction with RNA sequences [87] .

DNA topoisomerase I, Clk ,PI3K and the interaction of different SR proteins with exon 5 play a major role in exon 5 inclusion/exclusion as described in Table I and Table II.

Upstream regulator	Regulated SR proteins	exon 5 incluson/exclusion	Ref
DNA topoisomerase I	SRp55 and SF2/ASF	inclusion	[88]
PI3K	SRp55 and SF2/ASF ,SRp75	exclusion	[89]
Clk	SRp55 , SF2/ASF, SRp75, SRp40 and SRP20	inclusion, exclusion	[88]
NFκB		inclusion, exclusion	[89]

Table I. Upstream regulator pathways , their effects on SR protein phosphorylation and their effects on alternative splicing of the TF gene.

SR proteins	exon 5 inclusion/exclusion	Ref
SRp75 and SF2/ASF	exclusion	[88-90]
SF2/ASF and SRp55	inclusion	[91]
SRp40 and SRp35	exclusion	[92]

Table II: Unique sets of SR proteins and their effects on exon 5 inclusion/exclusion.

Alternatively Spliced Tissue Factor in Coagulation

The coagulant function of asTF is still under debate. The FVIIa binding site of flTF is conserved in asTF, and asTF localizes to thrombi [80]. Therefore, it is tempting to hypothesize that asTF plays a role in the coagulation. Indeed, addition of recombinant hasTF to platelet poor plasma decreases the clotting time and this pro-coagulant effect of asTF is dependent on the presence of phospholipids. [80]. Similarly, recombinant masTF showed coagulant activity in FXa generation assays [86]. In an other study,incubation of endothelial cells with cytokines resulted in hflTF and hasTF production while hasTF depletion reduced coagulant activity [83]. It should be noted that asTF is not tethered to the cell surface and the lack of such a phospholipid surface may compromise coagulant activity. Thus it remains unclear how asTF/FVIIa complex interacts with the cell surface in order to activate FX.

In contrast with the above, other studies using cell lysates or supernantant from HEK293 and Mia-PaCa-2 cells transfected to express hflTF and hasTF, show prominent coagulant activity only when cells were transfected to express flTF and only flTF could be detected in the culture media of these cells, which is in contradiction with asTF's secretable nature [93, 94]. While in all these previously mentioned experimental designs, asTF or flTF were expressed separately, more recent work in which flTF and asTF were co-expressed suggests that asTF enhances flTF's coagulant activity [95]. In these studies, asTF was co-localized with flTF antigen on both cell surfaces and microparticles. Although simultaneous TF isoform expression did not lead to any change in flTF levels on cells, TF activity was significantly higher on cells expressing both isoforms than that on cells harboring solely flTF [95]. Thus, it appears that asTF enhances flTF function and further studies to investigate the thrombogenicity of asTF are warranted.

Alternatively Spliced Tissue Factor Signaling

flTF-dependent PAR signaling is both supported and limited by their membrane tethered nature, as the majority of both flTF and PAR2 localize in lipid rafts. This domain allows the positioning of these proteins in close proximity to trigger downstream signaling events [68]. In contrast, non membrane-tethered asTF may not be optimally positioned on a membrane surface and does not facilitate FVIIa-dependent PAR2 activation. Rather, asTF ligates integrin subsets on the cell surface to activate signaling pathways [96]. Integrins are membrane receptors composed of an α and β subunit. 18 α and 8 β subunits associate to form 24 different integrin receptors. They are involved in cell-cell or cell-extracellular matrix interaction and inform the cells on surrounding conditons. Utilization of different integrin subsets by asTF results in the activation of various signaling pathways that regulate diverse cellular functions. On endothelial cells, hasTF/integrin interaction increases the phosphorylation of FAK, p42/44 MAPK, p38 MAPK and Akt. Particularly, αvβ3 ligation triggers endothelial cell migration whereas α6β1 is involved in capillary formation [96]. Although, masTF-induced sprout formation is dependent on β1 and β3 as well, endothelial cell adhesion is only mediated by β3 integrin [97]. The difference in integrin specificity between hasTF and masTF may be the result of the distinct C-termini. asTF also appears to play a substantial role in cancer progression. asTF levels correlate with lower survival and grade among non-small cell lung carcinoma patients [98, 99]. In addition, grafting of asTF- but not flTF- expressing pancreatic MiaPaCa cancer cells into mice yielded larger and more vascularized tumors [100].

Apart from cancer, asTF also appears to influence atherosclerotic progression. β1 integrin ligation upregulates the expression of adhesion molecules (i.e. E-selectin, ICAM-1, VCAM-1) on endothelial cells giving rise to enhanced binding and transmigration of monocytes [101]. Despite a role for asTF in pathology, asTF may also dampen progression of certain diseases. In mouse cardiomyocytes asTF activates a signaling cascade composed of αVβ3, Akt and NFκB. Activation of this cassette leads to increased expression of anti-apoptotic Bcl-XL and protects cardiomyocytes against TNF-α induced cell death [102]. Survival signals for the cardiomyocytes are vital due to their inability to proliferate. Therefore asTF might play a protective role against cardiac dysfunction by decreasing apoptosis rates.

Outline

In this thesis we aimed to elucidate the specific roles of TF isoforms in coagulation, cell proliferation and angiogenesis. To achieve these objectives, we utilized patient cohorts, *in vivo* and *in vitro* tools.

Chapter 2 outlines the current findings on the contribution of each TF isoform to coagulation, atherosclerosis, cancer progression and cancer associated thrombosis. **Chapter 3** investigates the possible mechanisms regulating flTF coagulant function, in particular the disulfide switch model and flTF dimerization. **Chapter 4** not only zooms into the TF regulated processes which were discussed in Chapter 2 but also summarizes the impact of integrin complexation on these processes. This chapter also gives further directions to improve our knowledge on TF/integrin signaling and disease progression. **Chapter 5** investigates the unique role of TF isoforms, specifically asTF, in breast cancer progression. The chapter is expanded by unveiling the mechanism lying behind asTF dependent tumor growth. The individual impact of each isoform was evaluated in **Chapter 6** is a side-by-side comparison study. The hormonal regulation of breast cancer prompted us to investigate the interaction of asTF and the estrogen pathway in **chapter 7**. Moreover, a widely used breast cancer model that was exploited in this thesis is thoroughly described in **Chapter 8**. Finally, **chapter 9** presents the general summary and discussion.

Reference List

[1] Beck EA. The chemistry of blood coagulation: a summary by Paul Morawitz (1905). Thromb Haemost 1977 Jun 30;37(3):376-9.

[2] Colucci M, Balconi G, Lorenzet R, Pietra A, Locati D, Donati MB, et al. Cultured human endothelial cells generate tissue factor in response to endotoxin. J Clin Invest 1983 Jun;71(6):1893-6.

[3] Drake TA, Cheng J, Chang A, Taylor FB, Jr. Expression of tissue factor, thrombomodulin, and E-selectin in baboons with lethal Escherichia coli sepsis. Am J Pathol 1993 May;142(5):1458-70.

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

[5] Song D, Ye X, Xu H, Liu SF. Activation of endothelial intrinsic NF-{kappa}B pathway impairs protein C anticoagulation mechanism and promotes coagulation in endotoxemic mice. Blood 2009 Sep 17;114(12):2521-9.

[6] Girard TJ, Warren LA, Novotny WF, Likert KM, Brown SG, Miletich JP, et al. Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. Nature 1989 Apr 6;338(6215):518-20.

[7] Dahlback B, Villoutreix BO. The anticoagulant protein C pathway. FEBS Lett 2005 Jun 13;579(15):3310-6.

[8] Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de RH, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994 May 5;369(6475):64-7.

[9] Vergnolle N. Protease-activated receptors and inflammatory hyperalgesia. Mem Inst Oswaldo Cruz 2005 Mar;100 Suppl 1:173-6.

[10] Mannaioni G, Orr AG, Hamill CE, Yuan H, Pedone KH, McCoy KL, et al. Plasmin potentiates synaptic N-methyl-D-aspartate receptor function in hippocampal neurons through activation of protease-activated receptor-1. J Biol Chem 2008 Jul 18;283(29):20600-11.

[11] Ludeman MJ, Kataoka H, Srinivasan Y, Esmon NL, Esmon CT, Coughlin SR. PAR1 cleavage and signaling in response to activated protein C and thrombin. J Biol Chem 2005 Apr 1;280(13):13122-8.

[12] Riewald M, Kravchenko VV, Petrovan RJ, O'Brien PJ, Brass LF, Ulevitch RJ, et al. Gene induction by coagulation factor Xa is mediated by activation of protease-activated receptor 1. Blood 2001 May 15;97(10):3109-16.

[13] Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, Kuliopulos A. PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. Cell 2005 Feb 11;120(3):303-13.

[14] Nystedt S, Emilsson K, Wahlestedt C, Sundelin J. Molecular cloning of a potential proteinase activated receptor. Proc Natl Acad Sci U S A 1994 Sep 27;91(20):9208-12.

[15] Molino M, Barnathan ES, Numerof R, Clark J, Dreyer M, Cumashi A, et al. Interactions of mast cell tryptase with thrombin receptors and PAR-2. J Biol Chem 1997 Feb 14;272(7):4043-9.

[16] Takeuchi T, Harris JL, Huang W, Yan KW, Coughlin SR, Craik CS. Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. J Biol Chem 2000 Aug 25;275(34):26333-42.

[17] Lourbakos A, Chinni C, Thompson P, Potempa J, Travis J, Mackie EJ, et al. Cleavage and activation of proteinase-activated receptor-2 on human neutrophils by gingipain-R from Porphyromonas gingivalis. FEBS Lett 1998 Sep 11;435(1):45-8.

[18] Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc Natl Acad Sci U S A 2000 May 9;97(10):5255-60.

[19] Riewald M, Ruf W. Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. Proc Natl Acad Sci U S A 2001 Jul 3;98(14):7742-7.

[20] Andersen H, Greenberg DL, Fujikawa K, Xu W, Chung DW, Davie EW. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. Proc Natl Acad Sci U S A 1999 Sep 28;96(20):11189-93.

[21] Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest 1999 Mar;103(6):879-87.

[22] Ishihara H, Zeng D, Connolly AJ, Tam C, Coughlin SR. Antibodies to protease-activated receptor 3 inhibit activation of mouse platelets by thrombin. Blood 1998 Jun 1;91(11):4152-7.

[23] Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. PAR3 is a cofactor for PAR4 activation by thrombin. Nature 2000 Apr 6;404(6778):609-13.

[24] O'Brien PJ, Prevost N, Molino M, Hollinger MK, Woolkalis MJ, Woulfe DS, et al. Thrombin responses in human endothelial cells. Contributions from receptors other than PAR1 include the transactivation of PAR2 by thrombin-cleaved PAR1. J Biol Chem 2000 May 5;275(18):13502-9.

[25] Schmidt VA, Nierman WC, Maglott DR, Cupit LD, Moskowitz KA, Wainer JA, et al. The human proteinase-activated receptor-3 (PAR-3) gene. Identification within a Par gene cluster and characterization in vascular endothelial cells and platelets. J Biol Chem 1998 Jun 12;273(24):15061-8.

[26] Storck J, Kusters B, Zimmermann ER. The tethered ligand receptor is the responsible receptor for the thrombin induced release of von Willebrand factor from endothelial cells (HUVEC). Thromb Res 1995 Feb 1;77(3):249-58.

[27] Bazzoni G. Endothelial tight junctions: permeable barriers of the vessel wall. Thromb Haemost 2006 Jan;95(1):36-42.

[28] Bae JS, Yang L, Manithody C, Rezaie AR. The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. Blood 2007 Dec 1;110(12):3909-16.

[29] Frenette PS, Mayadas TN, Rayburn H, Hynes RO, Wagner DD. Susceptibility to infection and altered hematopoiesis in mice deficient in both P- and E-selectins. Cell 1996 Feb 23;84(4):563-74.

[30] Hattori R, Hamilton KK, Fugate RD, McEver RP, Sims PJ. Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. J Biol Chem 1989 May 15;264(14):7768-71.

[31] Johnson K, Choi Y, DeGroot E, Samuels I, Creasey A, Aarden L. Potential mechanisms for a proinflammatory vascular cytokine response to coagulation activation. J Immunol 1998 May 15;160(10):5130-5.

[32] Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science 2002 Jun 7;296(5574):1880-2.

[33] Joyce DE, Gelbert L, Ciaccia A, DeHoff B, Grinnell BW. Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. J Biol Chem 2001 Apr 6;276(14):11199-203.

[34] Feistritzer C, Riewald M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. Blood 2005 Apr 15;105(8):3178-84.

[35] McLaughlin JN, Shen L, Holinstat M, Brooks JD, Dibenedetto E, Hamm HE. Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. J Biol Chem 2005 Jul 1;280(26):25048-59.

[36] Feistritzer C, Riewald M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. Blood 2005 Apr 15;105(8):3178-84.

[37] Versteeg HH, Schaffner F, Kerver M, Petersen HH, Ahamed J, Felding-Habermann B, et al. Inhibition of tissue factor signaling suppresses tumor growth. Blood 2008 Jan 1;111(1):190-9.

[38] Ahamed J, Ruf W. Protease-activated receptor 2-dependent phosphorylation of the tissue factor cytoplasmic domain. J Biol Chem 2004 May 28;279(22):23038-44.

[39] Ettelaie C, Elkeeb AM, Maraveyas A, Collier ME. p38alpha phosphorylates serine 258 within the cytoplasmic domain of tissue factor and prevents its incorporation into cell-derived microparticles. Biochim Biophys Acta 2013 Mar;1833(3):613-21.

[40] Versteeg HH, Spek CA, Slofstra SH, Diks SH, Richel DJ, Peppelenbosch MP. FVIIa:TF induces cell survival via G12/G13-dependent Jak/STAT activation and BclXL production. Circ Res 2004 Apr 30;94(8):1032-40.

[41] Camerer E, Gjernes E, Wiiger M, Pringle S, Prydz H. Binding of factor VIIa to tissue factor on keratinocytes induces gene expression. J Biol Chem 2000 Mar 3;275(9):6580-5.

[42] Sorensen BB, Freskgard PO, Nielsen LS, Rao LV, Ezban M, Petersen LC. Factor VIIa-induced p44/42 mitogen-activated protein kinase activation requires the proteolytic activity of factor VIIa and is independent of the tissue factor cytoplasmic domain. J Biol Chem 1999 Jul 23;274(30):21349-54.

[43] Ott I, Weigand B, Michl R, Seitz I, Sabbari-Erfani N, Neumann FJ, et al. Tissue factor cytoplasmic domain stimulates migration by activation of the GTPase Rac1 and the mitogen-activated protein kinase p38. Circulation 2005 Jan 25;111(3):349-55.

[44] Ott I, Fischer EG, Miyagi Y, Mueller BM, Ruf W. A role for tissue factor in cell adhesion and migration mediated by interaction with actin-binding protein 280. J Cell Biol 1998 Mar 9;140(5):1241-53.

[45] Belting M, Dorrell MI, Sandgren S, Aguilar E, Ahamed J, Dorfleutner A, et al. Regulation of angiogenesis by tissue factor cytoplasmic domain signaling. Nat Med 2004 May;10(5):502-9.

[46] Dorfleutner A, Hintermann E, Tarui T, Takada Y, Ruf W. Cross-talk of integrin alpha3beta1 and tissue factor in cell migration. Mol Biol Cell 2004 Oct;15(10):4416-25.

[47] Versteeg HH, Schaffner F, Kerver M, Ellies LG, Andrade-Gordon P, Mueller BM, et al. Protease-activated receptor (PAR) 2, but not PAR1, signaling promotes the development of mammary adenocarcinoma in polyoma middle T mice. Cancer Res 2008 Sep 1;68(17):7219-27.

[48] Hembrough TA, Swartz GM, Papathanassiu A, Vlasuk GP, Rote WE, Green SJ, et al. Tissue factor/factor VIIa inhibitors block angiogenesis and tumor growth through a nonhemostatic mechanism. Cancer Res 2003 Jun 1;63(11):2997-3000.

[49] Albrektsen T, Sorensen BB, Hjorto GM, Fleckner J, Rao LV, Petersen LC. Transcriptional program induced by factor VIIa-tissue factor, PAR1 and PAR2 in MDA-MB-231 cells. J Thromb Haemost 2007 Aug;5(8):1588-97.

[50] Hjortoe GM, Petersen LC, Albrektsen T, Sorensen BB, Norby PL, Mandal SK, et al. Tissue factor-factor VIIa-specific up-regulation of IL-8 expression in MDA-MB-231 cells is mediated by PAR-2 and results in increased cell migration. Blood 2004 Apr 15;103(8):3029-37.

[51] Liu Y, Mueller BM. Protease-activated receptor-2 regulates vascular endothelial growth factor expression in MDA-MB-231 cells via MAPK pathways. Biochem Biophys Res Commun 2006 Jun 16;344(4):1263-70.

[52] Regina S, Rollin J, Blechet C, Iochmann S, Reverdiau P, Gruel Y. Tissue factor expression in non-small cell lung cancer: relationship with vascular endothelial growth factor expression, microvascular density, and K-ras mutation. J Thorac Oncol 2008 Jul;3(7):689-97.

[53] Kakkar AK, Lemoine NR, Scully MF, Tebbutt S, Williamson RC. Tissue factor expression correlates with histological grade in human pancreatic cancer. Br J Surg 1995 Aug;82(8):1101-4.

[54] Abdulkadir SA, Carvalhal GF, Kaleem Z, Kisiel W, Humphrey PA, Catalona WJ, et al. Tissue factor expression and angiogenesis in human prostate carcinoma. Hum Pathol 2000 Apr;31(4):443-7.

[55] Guan M, Jin J, Su B, Liu WW, Lu Y. Tissue factor expression and angiogenesis in human glioma. Clin Biochem 2002 Jun;35(4):321-5.

[56] Taniguchi T, Kakkar AK, Tuddenham EG, Williamson RC, Lemoine NR. Enhanced expression of urokinase receptor induced through the tissue factor-factor VIIa pathway in human pancreatic cancer. Cancer Res 1998 Oct 1;58(19):4461-7.

[57] Taniguchi T, Lemoine NR, Kakkar AK. Urokinase receptor is a key player in tumour progression. Gut 1998 Dec;43(6):739-40.

[58] Fang J, Wei WN, Xia LH, Song SJ. [Study of the effect of TF/FVIIa complex on the expression of u-PAR mRNA in human ovarian cancer]. Zhonghua Xue Ye Xue Za Zhi 2004 Mar;25(3):143-6.

[59] Bromberg ME, Konigsberg WH, Madison JF, Pawashe A, Garen A. Tissue factor promotes melanoma metastasis by a pathway independent of blood coagulation. Proc Natl Acad Sci U S A 1995 Aug 29;92(18):8205-9.

[60] Fang J, Gu L, Zhu N, Tang H, Alvarado CS, Zhou M. Tissue factor/FVIIa activates Bcl-2 and prevents doxorubicin-induced apoptosis in neuroblastoma cells. BMC Cancer 2008;8:69.

[61] Arderiu G, Pena E, Aledo R, Juan-Babot O, Badimon L. Tissue factor regulates microvessel formation and stabilization by induction of chemokine (C-C motif) ligand 2 expression. Arterioscler Thromb Vasc Biol 2011 Nov;31(11):2607-15.

[62] Arderiu G, Pena E, Aledo R, Badimon L. Tissue factor-Akt signaling triggers microvessel formation. J Thromb Haemost 2012 Sep;10(9):1895-905.

[63] Ettelaie C, Li C, Collier ME, Pradier A, Frentzou GA, Wood CG, et al. Differential functions of tissue factor in the trans-activation of cellular signalling pathways. Atherosclerosis 2007 Sep;194(1):88-101.

[64] Bach RR. Tissue factor encryption. Arterioscler Thromb Vasc Biol 2006 Mar;26(3):456-61.

[65] Zwaal RF, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. Cell Mol Life Sci 2005 May;62(9):971-88.

[66] Kirszberg C, Lima LG, Da Silva de OA, Pickering W, Gray E, Barrowcliffe TW, et al. Simultaneous tissue factor expression and phosphatidylserine exposure account for the highly procoagulant pattern of melanoma cell lines. Melanoma Res 2009 Oct;19(5):301-8.

[67] Pike LJ. Lipid rafts: bringing order to chaos. J Lipid Res 2003 Apr;44(4):655-67.

[68] Awasthi V, Mandal SK, Papanna V, Rao LV, Pendurthi UR. Modulation of tissue factor-factor VIIa signaling by lipid rafts and caveolae. Arterioscler Thromb Vasc Biol 2007 Jun;27(6):1447-55.

[69] Henriquez S, Calderon C, Quezada M, Oliva B, Bravo ML, Aranda E, et al. Progesterone utilizes distinct membrane pools of tissue factor to increase coagulation and invasion and these effects are inhibited by TFPI. J Cell Physiol 2011 Dec;226(12):3278-85.

[70] Sevinsky JR, Rao LV, Ruf W. Ligand-induced protease receptor translocation into caveolae: a mechanism for regulating cell surface proteolysis of the tissue factor-dependent coagulation pathway. J Cell Biol 1996 Apr;133(2):293-304.

[71] Bach RR, Moldow CF. Mechanism of tissue factor activation on HL-60 cells. Blood 1997 May 1;89(9):3270-6.

[72] Wolberg AS, Monroe DM, Roberts HR, Hoffman MR. Tissue factor de-encryption: ionophore treatment induces changes in tissue factor activity by phosphatidylserine-dependent and -independent mechanisms. Blood Coagul Fibrinolysis 1999 Jun;10(4):201-10.

[73] Donate F, Kelly CR, Ruf W, Edgington TS. Dimerization of tissue factor supports solution-phase autoactivation of factor VII without influencing proteolytic activation of factor X. Biochemistry 2000 Sep 19;39(37):11467-76.

[74] Ahamed J, Versteeg HH, Kerver M, Chen VM, Mueller BM, Hogg PJ, et al. Disulfide isomerization switches tissue factor from coagulation to cell signaling. Proc Natl Acad Sci U S A 2006 Sep 19;103(38):13932-7.

[75] van den Hengel LG, Osanto S, Reitsma PH, Versteeg HH. Murine tissue factor coagulant activity is critically dependent on the presence of an intact allosteric disulfide. Haematologica 2013 Jan;98(1):153-8.

[76] Liang HP, Brophy TM, Hogg PJ. Redox properties of the tissue factor Cys186-Cys209 disulfide bond. Biochem J 2011 Aug 1;437(3):455-60.

[77] Rehemtulla A, Ruf W, Edgington TS. The integrity of the cysteine 186-cysteine 209 bond of the second disulfide loop of tissue factor is required for binding of factor VII. J Biol Chem 1991 Jun 5;266(16):10294-9.

[78] Reinhardt C, von Bruhl ML, Manukyan D, Grahl L, Lorenz M, Altmann B, et al. Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation. J Clin Invest 2008 Mar;118(3):1110-22.

[79] Kothari H, Nayak RC, Rao LV, Pendurthi UR. Cystine 186-cystine 209 disulfide bond is not essential for the procoagulant activity of tissue factor or for its de-encryption. Blood 2010 May 27;115(21):4273-83.

[80] Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. Nat Med 2003 Apr;9(4):458-62.

[81] Signaevsky M, Hobbs J, Doll J, Liu N, Soff GA. Role of alternatively spliced tissue factor in pancreatic cancer growth and angiogenesis. Semin Thromb Hemost 2008 Mar;34(2):161-9.

[82] Beuneu C, Vosters O, Movahedi B, Remmelink M, Salmon I, Pipeleers D, et al. Human pancreatic duct cells exert tissue factor-dependent procoagulant activity: relevance to islet transplantation. Diabetes 2004 Jun;53(6):1407-11.

[83] Szotowski B, Antoniak S, Poller W, Schultheiss HP, Rauch U. Procoagulant soluble tissue factor is released from endothelial cells in response to inflammatory cytokines. Circ Res 2005 Jun 24;96(12):1233-9.

[84] Yu JL, Rak JW. Shedding of tissue factor (TF)-containing microparticles rather than alternatively spliced TF is the main source of TF activity released from human cancer cells. J Thromb Haemost 2004 Nov;2(11):2065-7.

[85] Bruggemann LW, Drijfhout JW, Reitsma PH, Spek CA. Alternatively spliced tissue factor in mice: induction by Streptococcus pneumoniae. J Thromb Haemost 2006 Apr;4(4):918-20.

[86] Bogdanov VY, Kirk RI, Miller C, Hathcock JJ, Vele S, Gazdoiu M, et al. Identification and characterization of murine alternatively spliced tissue factor. J Thromb Haemost 2006 Jan;4(1):158-67.

[87] Eisenreich A, Rauch U. Regulation and differential role of the tissue factor isoforms in cardiovascular biology. Trends Cardiovasc Med 2010 Aug;20(6):199-203.

[88] Eisenreich A, Bogdanov VY, Zakrzewicz A, Pries A, Antoniak S, Poller W, et al. Cdc2-like kinases and DNA topoisomerase I regulate alternative splicing of tissue factor in human endothelial cells. Circ Res 2009 Mar 13;104(5):589-99.

[89] Eisenreich A, Malz R, Pepke W, Ayral Y, Poller W, Schultheiss HP, et al. Role of the phosphatidylinositol 3-kinase/protein kinase B pathway in regulating alternative splicing of tissue factor mRNA in human endothelial cells. Circ J 2009 Sep;73(9):1746-52.

[90] Fedorov O, Huber K, Eisenreich A, Filippakopoulos P, King O, Bullock AN, et al. Specific CLK inhibitors from a novel chemotype for regulation of alternative splicing. Chem Biol 2011 Jan 28;18(1):67-76.

[91] Tardos JG, Eisenreich A, Deikus G, Bechhofer DH, Chandradas S, Zafar U, et al. SR proteins ASF/SF2 and SRp55 participate in tissue factor biosynthesis in human monocytic cells. J Thromb Haemost 2008 May;6(5):877-84.

[92] Chandradas S, Deikus G, Tardos JG, Bogdanov VY. Antagonistic roles of four SR proteins in the biosynthesis of alternatively spliced tissue factor transcripts in monocytic cells. J Leukoc Biol 2010 Jan;87(1):147-52.

[93] Boing AN, Hau CM, Sturk A, Nieuwland R. Human alternatively spliced tissue factor is not secreted and does not trigger coagulation. J Thromb Haemost 2009 Aug;7(8):1423-6.

[94] Censarek P, Bobbe A, Grandoch M, Schror K, Weber AA. Alternatively spliced human tissue factor (asHTF) is not pro-coagulant. Thromb Haemost 2007 Jan;97(1):11-4.

[95] Unruh D, Turner K, Srinivasan R, Kocaturk B, Qi X, Chu Z, et al. Alternatively spliced tissue factor contributes to tumor spread and activation of coagulation in pancreatic ductal adenocarcinoma. Int J Cancer 2014 Jan 1;134(1):9-20.

[96] van den Berg YW, van den Hengel LG, Myers HR, Ayachi O, Jordanova E, Ruf W, et al. Alternatively spliced tissue factor induces angiogenesis through integrin ligation. Proc Natl Acad Sci U S A 2009 Nov 17;106(46):19497-502.

[97] Godby RC, van den Berg YW, Srinivasan R, Sturm R, Hui DY, Konieczny SF, et al. Nonproteolytic properties of murine alternatively spliced tissue factor: implications for integrin-mediated signaling in murine models. Mol Med 2012;18:771-9.

[98] Goldin-Lang P, Tran QV, Fichtner I, Eisenreich A, Antoniak S, Schulze K, et al. Tissue factor expression pattern in human non-small cell lung cancer tissues indicate increased blood thrombogenicity and tumor metastasis. Oncol Rep 2008 Jul;20(1):123-8.

[99] Rollin J, Regina S, Gruel Y. Tumor expression of alternatively spliced tissue factor is a prognostic marker in non-small cell lung cancer. J Thromb Haemost 2010 Mar;8(3):607-10.

[100] Hobbs JE, Zakarija A, Cundiff DL, Doll JA, Hymen E, Cornwell M, et al. Alternatively spliced human tissue factor promotes tumor growth and angiogenesis in a pancreatic cancer tumor model. Thromb Res 2007;120 Suppl 2:S13-S21.

[101] Srinivasan R, Ozhegov E, van den Berg YW, Aronow BJ, Franco RS, Palascak MB, et al. Splice variants of tissue factor promote monocyte-endothelial interactions by triggering the expression of cell adhesion molecules via integrin-mediated signaling. J Thromb Haemost 2011 Oct;9(10):2087-96.

[102] Boltzen U, Eisenreich A, Antoniak S, Weithaeuser A, Fechner H, Poller W, et al. Alternatively spliced tissue factor and full-length tissue factor protect cardiomyocytes against TNF-alpha-induced apoptosis. J Mol Cell Cardiol 2012 May;52(5):1056-65.