Chapter 9 – General Discussion and Summary

Background

From the simplest vertebrates to the most complex, the coagulation system is optimally fine-tuned to produce a common endpoint: formation of a gelatinous, fibrin rich structure that acts as a vascular plug to minimize blood loss upon damage to the blood vessel [1]. Serine protease cascades form the basis of many crucial systems in the body, including hemostasis [2]. In the coagulation cascade, the activation of each downstream element is mediated by an upstream factor thus enabling a tight regulation [3]. This control system is crucial as excessive activation of coagulation leads to thrombotic complications whereas diminished activation results in bleeding problems.

The coagulation cascade is divided into two connected pathways: the extrinsic and intrinsic pathway. The initiator of the extrinsic pathway is full length Tissue Factor (flTF) which comes into contact with circulating coagulation factors upon vessel damage. The interaction of flTF with blood activates a cascade of activation processes that eventually result in the formation of thrombin. In the intrinsic pathway, the low levels of thrombin are increased by FXIa, the tenase and pro-thrombinase complex on the platelet surface. Eventually, a fibrin mesh is formed to plug the damaged site. Although the contribution of both pathways in coagulation is indispensable, in this thesis emphasis is put on the molecular biology of the initiator of the extrinsic pathway, flTF.

Apart from being the major initiator of coagulation, flTF also has non-hemostatic properties. By forming a complex with FVIIa or FVIIa/FXa, it facilitates cellular signaling via PARs [4, 5]. In addition, flTF-dependent PAR signaling is further supported by the complexation of flTF with integrins [6]. Particularly on cancer cells, flTF signaling contributes to tumorigenic processes such as angiogenesis, tumor cell migration and survival [7, 8]. Therefore studies aimed at stemming cancer progression have begun to concentrate on the blockade of flTF-specific cellular signaling [6].

The majority of the membrane localized flTF exists in a cryptic state that has low FVIIa affinity and does not bind FX [9]. However, stimulation of cells with particular agents (i.e. Hg^{2+}, calcium ionophore) has a remarkable inducing effect on flTF coagulant activity [10, 11]. These data indicate the presence of regulatory mechanisms controlling the hemostatic capacity of flTF. Several models have been suggested to explain the decryption process. The first and most widely accepted one is the phosphatidylserine (PS) exposure model. According to this model, the asymmetrical cell membrane starts exposing PS on the outer leaflet by the action of scramblases. This results in the formation of a negatively charged surface that allows the optimal binding of coagulation factors [12]. In a second model, localization of flTF in lipid rafts is considered to be the major determinant of flTF

activation [13, 14]. The third model suggests that TF dimerization leads to decryption [15]. Last, the oxidoreductase Protein Disulfide Isomerase (PDI) may modulate an allosteric disulfide bond in flTF, leading to a switch between coagulant (oxidized) or signaling (reduced) flTF [9]. In the first part of this thesis, we investigated the validity of the disulfide switch model in the regulation of flTF coagulant function. We deemed this of importance, as the tight balance between coagulant and signaling TF may be deregulated in several diseases (i.e cancer, sepsis). Additionally, we analyzed the effect of flTF dimerization on TF clotting activity.

Ten years ago, another TF isoform resulting from the exclusion of exon 5 in the TF pre-mRNA has been described and named alternatively spliced Tissue Factor (asTF) [16]. Exclusion of exon 5 results in an mRNA frameshift and a concomitant change in amino acid composition of the C-terminal domain. asTF does not contain a transmembrane domain, but is a soluble protein. Although at present studies attribute a pro-angiogenic role to asTF, research regarding its function in a pathophysiological context is scarce. In the second part of this thesis, we aimed to fill the knowledge gap with respect to the involvement of asTF in cancer progression. Furthermore, we performed a side-by-side comparison of flTF-dependent and asTF-dependent effects in the same context.

Regulation of full length Tissue Factor activity

According to classical concepts, the membrane-bound nature of flTF restricts its action to the surface of andothelial cells. However, recent studies show that flTF is also detected in the circulation either on blood cells or as a soluble antigen. Additionally, in disease conditions such as sepsis or cancer, cells are triggered to release submicron vesicles called microparticles into the blood [17]. These blood-borne structures frequently expose flTF and may form another level of TF-dependent coagulation activation. It is possible that the direct interaction of MP-exposed flTF with coagulation factors that reside in the bloodstream causes thrombotic complications. In support of this, flTF-positive microparticles released from cancer cells are associated with higher thrombotic risk in a number of patient cohorts [18, 19]. There are also studies showing the lack of association between increased TF-exposing microparticle levels and venous thromboembolism (VTE) [20]. Thus, it is clear that there are also other factors causing elevated thrombotic risk. Although endogenous levels of blood borne TF exist under physiological conditions these levels do usually not activate coagulation, most likely due to its cryptic nature [21]. These results again underline the importance of proper decryption flTF models in coagulation and thrombosis (reviewed in **Chapter 2**).

Among the decryption models, the disulfide switch model has been subject to extensive debate, and a number of reports did not find evidence for the involvement of the TF allosteric disulfide in TF regulation [22, 23]. A paper by Kothari et al. reported that TF mutants that were previously used to mimic cryptic TF have similar procoagulant activity when compared to wild type (WT) TF. It is important to note that to mimic reduced human flTF, cysteine residues on position 186 and 209 are usually replaced with Ser or Ala, thus disrupting the disulfide. These mutations appear to mainly result in either protein misfolding -which causes their targeting for lysosomal degradation- or TF dimerization [9]. Thus, studies on the validity of disulfide switching as a mechanism to control TF decryption suffer from an inability to express TF mutants to the same extent as wild type (WT) TF [22]. In order to make the comparison possible, Kothari and colleagues calculated the coagulant activity per TF molecule. However, unequal expression of WT and mutant TF may have resulted in other artefacts that led these researchers to draw unsupported conclusions. In **Chapter 3**, we created BHK cells expressing similar 'total' and 'membrane-resident' protein levels of WT, 209A, 209S or 186/209S flTF. We did not observe proper membrane exposure of the C186S mutant, as shown before [9], most likely due to proteosomal degradation. Moreover, although the levels of WT flTF and the 209A, 209S and 186/209S mutants were similar, these mutants showed strongly reduced pro-coagulant function. Interestingly, we observed presence of Cys 186-linked flTF homodimers. Incubation of cells with a free thiol blocking reagent (NEM) disrupted dimer formation and decreased Xa generation suggesting higher procoagulant activity of the flTF dimers. Contradictory to our findings, Kothari et al. detected flTF dimers using mutants lacking both C186 and C209, which indicates the involvement of other, non-covalent bonds directing flTF dimerization in their experimental setup. It is important to note that we observed high coagulant activity within the TF dimer fraction, thus Kothari's conclusions may be based on an overestimation of TF mutant pro-coagulant activity .

So far, the disulfide switch model has only been supported *in vitro*. The validity of this model would definitely benefit from the development of proper models that test the role of TF disulfide switching *in vivo*. Mice expressing similar levels of WT flTF and reduced flTF may be subjected to tail bleeding tests or inferior *vena cava* ligations to evaluate the role of the TF disulfide in coagulation and thrombosis, respectively. In a pathophysiological context, plasma from cancer patients often contains higher concentrations of flTF-positive microparticles, and this has been suggested to confer a higher risk of VTE [18, 19, 24, 25]. It may also be of interest to evaluate the redox state of MP-flTF in cancer patients with and without a diagnosis of VTE.

Tissue Factor İsoforms in Cancer Progression

Initial studies have suggested that asTF contains procoagulant activity [16]. Subsequent studies have presented contrasting findings [26, 27]. These data encouraged investigations into the signaling properties of asTF. Our group previously showed that asTF targets integrins rather than PARs to induce downstream signaling events [28]. The same study also revealed that asTF enhances endothelial cell migration and capillary formation which, in combination, lead to angiogenesis [28]. Since angiogenesis is an important prerequisite for cancer progression, a role for asTF in cancer appeared likely. Subcutaneous xenografting of pancreatic cancer cells (Mia-Paca-2) expressing either asTF or flTF into mice resulted in larger and more vascularized tumors only upon asTF expression [29]. However, this work suffered from a lack of mechanistic insight as to the exact role of asTF in tumor growth and did not investigate whether asTF function in mouse models of tumor growth can be extrapolated to human disease.

Therefore, in **chapter 5**, we aimed to eliminate the knowledge gap regarding the role of asTF in a breast cancer setting. First, we evaluated the impact of asTF in human disease. We used a tissue microarray consisting of specimens from 574 breast cancer patients. Both flTF and asTF associated with tumor grade but asTF additionally associated with tumor size. Next, we grafted MCF-7 cells transfected with control, flTF or asTF cDNA into the mammary fat of mice. The details of the orthotopic grafting, as well as its benefits over subcutaneous grafting are extensively described in **chapter 8.** Our results are in line with the findings of Hobbs and colleagues : in an *in vitro* setting flTF expression conferred a proliferative advantage on breast cancer cells and immunohistochemistry suggested that this was due to higher proliferation rates of asTF-expressing cells compared to flTF-expressing cells and control cells. Moreover, asTF expression resulted in higher vascular density and more extensive macrophage infiltration. Both Mia-Paca-2 and MCF-7 cells express low levels of the TF:FVIIa receptor PAR-2 [30, 31] which may explain why flTF-expressing tumors failed to expand [6]. Although this appears to be in contrast with *in vitro* experiments showing that incubation of flTF cells with a signaling inhibiting antibody (10H10) decreases proliferation, we speculate that in these cells 10H10 disrupts flTF/integrin interaction to block proliferation [6]. Nevertheless, *in vivo* PAR2 signaling may prevail over TF/integrin interaction.

One caveat in our cell model is that asTF and flTF were not simultaneously expressed while under both physiological and pathological conditions, asTF and flTF are expressed simultaneously. To circumvent these challenges we next used an aggressive subclone of the MDA-MB-231 breast cancer cell line that -in contrast to the parent cell line- expresses ample amounts of asTF. Pro-angiogenic effects of asTF were described before [28], but our

in vitro experiments indicated that asTF-dependent tumor expansion results from enhanced tumor cell proliferation rather than vessel formation. In support of this, asTF expression coincided with decreased expression levels of negative regulators of cell cycle (i.e. $p27^{KIP}$, $p21^{WAF}$) and upregulated levels of positive regulators (i.e. CCNA1). In brief, these findings suggest that flTF and asTF use different signaling pathways (PAR and integrin respectively) and have distinct functions in the tumor environment.

In our *in vivo* setup, asTF expression yielded larger tumors with a higher vascular density. Although our *in vitro* work attributes a mitogenic role to asTF, other papers show that asTF is pro-angiogenic in nature [28]. Therefore, it has remained obscure whether asTF induces cell proliferation as a result of increased angiogenesis or whether asTF-dependent angiogenesis is a resultant of larger tumor size. In previous work, the angiogenic properties of asTF were assessed using exogenous recombinant asTF [28]. However, asTF might regulate other signaling pathways in cells to release additional regulators. Therefore, it would be interesting to perform angiogenesis assays in the presence of asTF-induced cellular releasates (e.g. supernatants from cancer cells treated with asTF) as an alternative to asTF exclusively.

Alternatively Spliced Tissue Factor and Integrins

Previous studies have shown that flTF/integrin complexation potentiates PAR activation [6]. Interestingly, asTF regulated signaling events are independent of PARs. asTF ligates αvβ3 integrins to activate p38 and PI3K/Akt pathway in the regulation of endothelial cell migration whereas binding to α6β1 enhances capillary formation via p42/44 and PI3K [28]. masTF uses a similar repertoire of integrins, but capillary formation in this setting requires both β1 and β3 integrins [32]. A detailed review covering TF isoform/integrin interaction in cellular processes and diseases is provided in **Chapter 4**.

The strict dependence of asTF effects on integrins prompted us to investigate if asTF/integrin interaction similarly promoted breast cancer progression (**Chapter 5)**. We showed that asTF–induced proliferation was dependent on β1 integrins in MCF-7 cells whereas β3 was not required in this cell type. Of note, β3 levels are low in MCF-7 cells [33] and this might explain the lack of β3-dependent asTF effects. Therefore we used another breast cancer cell line (MDA-MB-231mfp) which expresses both integrin subsets. In this cell line, asTF-dependent proliferation was also exclusively dependent on β1 integrins. By using immunofluorescent cell staining, we demonstrated that asTF not only binds, but also activates β1 integrins. To find a mechanistical explanation for the activation effect, we used a β1 antibody that is reactive to the region encompassing the membrane-proximal β-tail domain as well as a peptide representing aminoacids 579-799 which reside

within the same domain. The β-tail domain (βTD), which is located at the membrane proximal region, regulates integrin substrate binding. It is connected to the βA domain in the head region via the CD loop. The close interaction of the βTD with the βA domain keeps the integrin in a bent, inactive form. Upon substrate binding to the βTD, the release of the CD loop separates the two domains from each other resulting in a non-bent conformation and the activation of integrins [34-36]. Incubation of asTF cells either with antibody or peptide not only disrupted asTF/β1 integrin interaction but also hampered the proliferative effect of asTF. Moreover, we showed that extracellular asTF rather than intracellular asTF causes cell proliferation. Overall, we hypothesize that breast cancer cells secrete asTF which in turn binds to the βTD on integrins. As a result, the CD loop rearranges to activate integrins. Activated integrins become prone to extracellular matrix binding [35] which is known to regulate cell cycle by upregulating positive regulators (Cyclins) and downregulating the negative regulators ($p27^{Kip}$) [35, 37], which is exactly what we have observed in asTF-expressing cells.

As asTF produces its effects through integrins, it would have been of interest to silence β1 integrin in asTF-expressing cells and monitor effects *in vivo*. However, β1 integrins play pleiotropic roles in cancer progression and we deemed such an approach unfeasible. It would, in our view, be more feasible to make asTF mutants lacking the β1 integrin binding domain to investigate the cancer progression over time.

Tissue Factor Isoforms have discrete characteristics in the regulation of breast cancer

Accumulating data suggest a different pattern of action by flTF and asTF in breast cancer progression. The flTF/PAR axis impacts pro-angiogenic cytokine secretion via TF-ct phosphorylation and concomitant tumor growth [6, 38]. In **Chapter 5**, we observed that asTF induces cancer cell growth independent of angiogenesis. Therefore we hypothesized that flTF promotes tumor growth via angiogenesis whereas asTF triggers tumor cell proliferation. We used MCF-7 cells expressing either of the two isoforms. The low levels of PAR2 on these cells made it difficult to draw a conclusion on the relative inputs of flTF/PAR and asTF/integrin signaling in breast cancer progression. To tackle this problem, in **Chapter 6** we used MDA-MB-231mfp cells expressing all of the required elements (i.e flTF, asTF, PAR2 and integrins). We injected these cells orthotopically into mice in the presence of a flTF signaling blocking antibody (10H10) or an asTF blocking antibody (Rb1). In order to uncover if these two pathways have common downstream elements we also blocked TF isoforms simultaneously.

The inhibition of either asTF or flTF resulted in decreased tumor size. Combined blockade significantly decreased the tumor size further compared to 10H10 treatment while a trend

was observed compared to Rb1 treatment alone (p=0.12). These data underline that both isoforms potentially contribute to breast tumor progression but have distinct roles in the process. The lack of a significant difference between combination treatment and Rb1 treatment also hints at the presence of shared downstream components. We also investigated the role of TF isoforms in metastasis. The sole inhibition of flTF or asTF decreased metastatic load in the lungs whereas the combination only showed a trend towards more efficient inhibition of metastasis. The impact of asTF on metastatic spread was described before [39, 40]. Interestingly, the anti-metastatic effects caused by 10H10 blockade is in stark contrast with other publications [6]. We would like to note that the methodology used in our work and in previous papers differs: we have used an orthotopic xenograft model, while previous papers have detailed the effects of 10H10-mediated blockade on metastasis using a tail vein model. Our model, which is described in **Chapter 8**, recapitulates the entire metastasis process whereas the tail vein model only mimics the process post-intravasation. Thus flTF signaling may be important for the early phase of metastasis.

Alternatively Spliced Tissue Factor Synergizes with Estrogen Signaling Pathway to stimulate breast cancer progression

The use of an estrogen receptor (ER) positive (MCF-7) and ER negative cell line (MDA-MB-231mfp) in **Chapter 5** helped us to provide a mechanism regarding the contribution of asTF to breast cancer. In this context it is notable that ER-positive and -negative breast tumors require distinct therapies [41, 42] which indicates the presence of different pathways in ER+ and ER- breast cancer progression. With this in mind, in **Chapter 7** we designed a study to investigate if TF isoforms interact with the ER pathway to regulate breast cancer progression. We used the same tissue microarray as that described in **Chapter 5.** In **Chapter 7** we subdivided the patients into ER+ and ER- groups. We observed an association between asTF and grade/tumor size only in ER+ tumors. Next, we used the gene profiles obtained from microarray analyses in **Chapter 5** to identify similarities between asTF signaling pathways and other pathways. Ingenuity pathway analysis revealed that both flTF and asTF regulate genes that are also regulated by the ER pathway, particularly those that function in processes like cell proliferation and movement. By using MTT assays we showed that both flTF- and asTF-expressing cells show increased proliferation rates upon estradiol (E2) treatment. Nevertheless, asTF-expressing cells show an increased sensitivity to E2 stimulation when compared to flTF-expressing cells that is again dependent on asTF/integrin complexation. Of note, this proliferative advantage induced by the expression of TF isoforms were first identified in experiments using standard media (chapter 5) but were not observed in phenol red free (estrogen negative) media **(Chapter 7).** This is explained by the fact that phenol red acts as a weak stimulant of

the ER pathway. E2-induced asTF dependent tumor cell proliferation was also recapitulated in an *in vivo* setting by the subcutaneous implantation of E2 pellets. In wound scratch assays, asTF cells migrated faster compared to control cells. Addition of E2 increased migration rates to a similar extent in asTF and control cells which indicates that asTF and E2 do not synergize in cellular movement.

Although we have not been able to provide conclusive data, we hypothesize that both flTF and asTF induce downstream signaling events that further potentiate ER signaling, or vice versa. Another possible explanation might be the expression of a distinct subset of proliferative gene products by each pathway, that in combination potently induce proliferation. In our experimental setup we approached the asTF/ER synergy only from an asTF point-of-view. It would also be of interest to repeat the same type of experiments after silencing of the ER gene, considering the fact that we also observed asTF-dependent proliferation effects in an ER- cell line.

Conclusion

This thesis contributes to filling the knowledge gaps in the literature regarding the role of TF isoforms in cancer and coagulation. All studies described so far focused on targeting flTF in cancer therapy but neglected asTF's contribution to breast cancer growth. The findings described in this thesis have several implications. First, we showed that an association exists between asTF levels on one hand, and breast tumor size and grade on the other. This makes it tempting to suggest that asTF in plasma of breast cancer patients could serve as a diagnostic or prognostic factor. In addition, the functions of asTF in breast tumor growth may be targeted to reduce primary tumor growth. It must be mentioned that targeting asTF appears perfectly feasible, as asTF is non-coagulant and targeting asTF wil likely not compromise normal hemostasis. Third, side by side comparison studies highlight the distinct effects of asTF and flTF on cancer progression suggesting that simultaneous blockade of both isoforms may present an efficient therapeutic strategy. Finally, we also discovered a previously unknown synergistic effect of asTF with the estrogen receptor pathway that may change our view of hormonal regulation of breast cancer. We showed that asTF and estrogen receptor-dependent pathways converge at a downstream point to fuel breast tumor growth. Therefore we propose that in patients with breast tumors that express both asTF and estrogen receptor, inhibiting a common downstream element has a larger impact on tumor growth than blocking solely asTF or ER. **(Fig. 1)**.

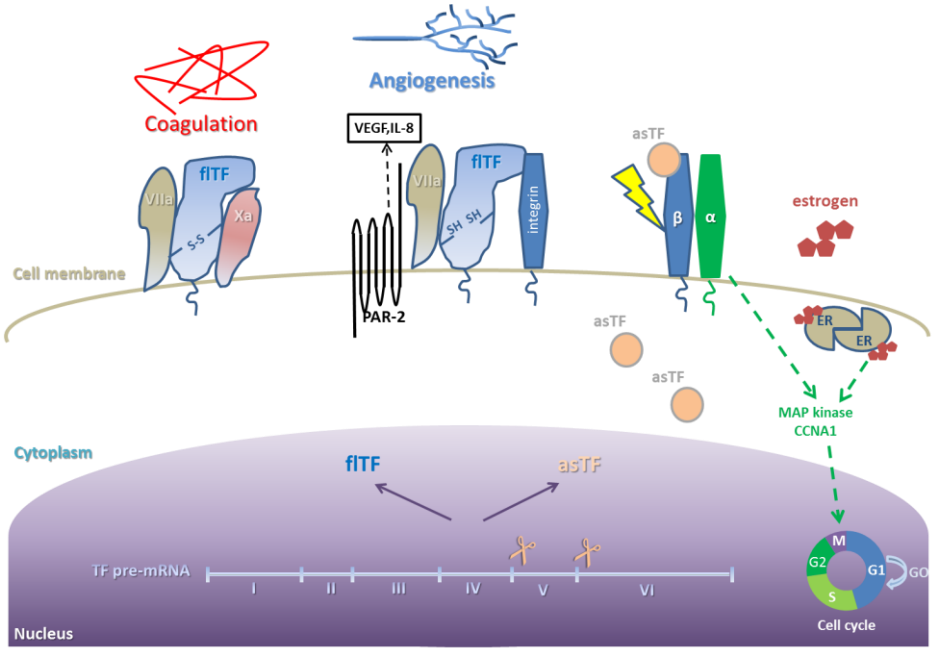

Fig.1. A schematic representation of the findings described in this thesis. Oxidation of the Cys186-Cys209 disulfide bond results in pro-coagulant tissue factor that has high affinity for FVIIa and capability to generate FXa. Reduction of the disulfide results in the formation of a non-coagulant TF pool that acts as a signaling molecule by facilitating the activation of PAR2 by FVIIa. Upon flTF-dependent PAR2 signaling pro-angiogenic cytokines are released which in turn trigger angiogenesis. The increased vascular density in the tumor milieu results in tumor growth. Alternative splicing of TF pre-mRNA causes the exclusion of exon 5. The newly formed antigen is soluble thus can be secreted and bound to integrins. Integrin ligation (in particular β1) by asTF downregulates cell cycle inhibitors whereas positive regulators are increased. Thus asTF-positive breast tumors expand at a highjer rate compared to asTF-negative tumors. Synergiy with the estrogen pathway, confers an even stronger mitogenic activity to asTF . This underlines the potential role of hormonal status in asTF-dependent proliferation.

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