Tissue factor isoforms and factor VII in cancer progression

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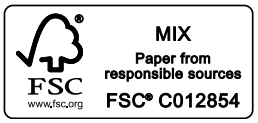

Voor mijn ouders en broer

Voor Ana

Contents

Chapter 1 – General introduction and outline of this thesis

Ever since the 19th century the co-occurrence of cancer and venous thromboembolism (VTE) has been observed, most commonly referred to as Trousseau's syndrome.[1] A malignancy increases VTE risk,[2] although VTE incidence differs per tumor type: pancreatic, brain and haematological malignancies are associated with the highest incidences.[3] Although the exact mechanism behind Trousseau's syndrome is still unknown, the hyper-coagulable state of tumor cells as a result of Tissue Factor (TF) expression has been widely established.[4]

Full length TF (flTF), the initiatior of extrinsic coagulation, is a 47-kDa transmembrane glycoprotein expressed on the subendothelium, acting as a hemostatic envelope surrounding organs and tissue. After blood vessel damage flTF is exposed to circulation-derived Factor VII (FVII), which it binds and activates. The subsequent flTF:FVIIa complex can bind and activate Factor X (FX), ultimately leading to thrombin generation and clot formation.[5] FlTF is expressed in various tumor types and associated with worsened clinical outcome.[6] Besides coagulation activation, flTF:FVIIa can activate the protease activated receptor 2 (PAR2), which ultimately leads to increased angiogenesis.[7,8]

In 2003, Bogdanov *et al*. reported on a soluble splice variant of TF which lacks the transmembrane region and exhibits a unique C-terminal tail as a result of a frameshift.[9] Alternatively spliced TF (asTF) is non-coagulant[10], but can induce angiogenesis and tumor growth via integrin ligation, independently of FVII or PAR2.[11,12] In recent years, it has been reported that tumor cells can ectopically produce FVII *in vitro* through hypoxia-inducable factor 2α (HIF-2α) or via EGRF-dependent pathways.[13,14] Furthermore, FVII expression enhanced cell migration and invasion in a TF-dependent manner.[15]

Outline

In this thesis, the relative role of TF isoforms and FVII was investigated in breast, bone and colorectal cancer patient cohorts. Furthermore, in breast and bone cancer, a multitude of experimental *in vitro* and *in vivo* models were used to demonstrate that TF isoform inhibition severely attenuated oncogenic processes.

Chapter 2 reviews the current knowledge of the effects of anticoagulants on oncologic processes in both patients and experimental cancer models. Whereas anticoagulants routinely given as thrombosis prophylaxis are reported as having a mixed to modest influence in attenuating cancer progression, a sizable body of evidence is reported on anticoagulant anti-cancer effects in a multitude of *in vitro* and *in vivo* models. In **Chapter 3** asTF but not flTF is strongly associated with tumor size and grade in a large breast cancer cohort. AsTF expression induces tumor cell proliferation by binding to β1 integrins, promotes oncogenic gene expression and tumor expansion *in vivo*. Also, asTF blockade strongly reduced *in vivo* tumor growth. **Chapter 4** continued with this study, showing that asTF expression was associated with tumor stage and grade in Estrogen receptor (ER) positive tumors only. AsTF expression combined with estradiol exposure resulted in a synergetic increase in *in vitro* and *in vivo* tumor expansion. Investigating the same breast cancer cohort, **Chapter 5** provides evidence that ectopic FVII expression is associated with reduced survival and higher tumor grade. FlTF and FVII co-expression drives tumor cell migration and invasion via PAR2 signaling, and ectopic FVII expression increased *in vivo* tumor expansion and liver metastasis independently of circulation FVII. **Chapter 6** is the first report of TF expression in an osteosarcoma cohort. High TF expression was associated with reduced patient survival and Inhibition of TF reduced both expression of pro-angiogenic factors as well as tumor cell invasiveness. TF blockade significantly reduced *in vivo* tumor expansion as well as lung metastasis. **Chapter 7** reports on flTF, asTF and FVII expression in a large colorectal cancer cohort: flTF, but not asTF, was associated with tumor stage and patient survival. FVII was widely

expressed and was associated with mucinous tumors and micro-satellite instability but not survival. **Chapter 8** provides a general summary and discussion on this thesis.

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Chapter 2 – Anticoagulants versus Cancer

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Abstract

Venous thromboembolism (VTE) and cancer are strongly associated, and present a major challenge in cancer patient treatment. Cancer patients have a higher risk of developing VTE, although the differs widely among tumour type. VTE prophylaxis is routinely given to cancer patients, in the form of vitamin K antagonists (VKA) or low molecular weight heparin (LMWH). Several studies have reported that cancer patients receiving anticoagulants show prolonged survival and this effect was more pronounced in patients with a good prognosis, although the mechanism is poorly understood. Tissue Factor (TF) is the initiator of extrinsic coagulation, but its non-haemostatic signalling via protease activated receptors (PARs) is a potent driver of tumour angiogenesis. Furthermore, coagulation activation is strongly implicated in tumour cell migration and metastasis. This review described the literature on anticoagulants and whether they inhibit cancer progression in patients, as well as inhibition of tumour cell proliferation, angiogenesis, and metastasis in both *in vitro* and *in vivo* models. Inhibition of TF signalling shows great promise in curbing angiogenesis and *in vivo* tumour growth, but whether this translates to patients is not yet known. Furthermore, non-haemostatic properties of coagulation factors in cancer progression are discussed, which provide exciting opportunities on limiting oncologic processes without affecting blood coagulation.

Introduction

Ever since the 19th century an association between cancer and venous thromboembolism (VTE) has been observed, presenting a major challenge in the treatment of cancer patients.[1] Malignancy increases the risk of VTE 4.1-fold, which increases even further to 6.5-fold in patients receiving chemotherapy.[2] VTE incidences differ per cancer type, with pancreatic, brain and haematological malignancies resulting in the highest incidences.[3] Treatment of VTE has consisted of unfractioned heparin (UFH), now largely replaced by low-molecular-weight heparins (LMWH) or vitamin K antagonists. Cancer patients that develop a VTE and receive LMWHs or

VKAs are at an increased risk of recurrent VTE as well as bleeding complications.[45] Extensive studies have been performed to investigate how VTE incidence can be reduced and whether VTE treatment might have any influence on cancer progression and overall patient survival.[6,7,8]
The development of experimental animal models in the second half of the 20th century allowed for the investigation of anticoagulants on experimental tumour development. Several *in vivo* studies have shown that tumour progression in these models is hampered by vitamin K antagonists (VKAs).[9]
VTE is caused by the activation of the blood coagulation cascade. Multiple risk factors (besides active cancer) such as surgery, immobility and age have been identified.[10] Extrinsic coagulation is initiated when Tissue Factor (TF), a transmembrane glycoprotein expressed on the subendothelium of vessels, comes into contact with Factor VII (FVII), which is activated upon binding. The TF:FVIIa complex can bind and activate Factor X (FX), which can activate Prothrombin (FII) to thrombin. Intrinsic coagulation involves subsequent activation of Factors XII, XI, IX and X, resulting in thrombin generation. Nevertheless, it must be mentioned that *in vivo*, TF is the sole activator of the coagulation cascade with the intrinsic pathway serving as an amplifier or thrombin generation.[11] Thrombin activates platelets as well as fibrinogen, leading to stable fibrin clots. Coagulation activation also leads to activation of coagulation inhibitors to limit the scope and duration of clot formation. Antithrombin, a serine protease inhibitor, is able to inactivate thrombin, FXa and FIXa, and this can be accelerated by heparin sulphate proteoglycans. LMWHs, routinely used for VTE prophylaxis, accelerate the ability of antithrombin to inactivate FXa, thus inhibiting coagulation.[12]
Tissue Factor Pathway Inhibitor (TFPI) is an inhibitor of both the TF:FVIIa complex as well as FXa.[13] Carboxylation of Factors II, VII, IX and X is dependent on vitamin K, which is oxidized in the process. Vitamin K epoxide reductase (VKORC) reduces vitamin K, thus repleting vitamin K available for coagulation. VKAs block the reduction of vitamin K, leading to a shortage of carboxylated coagulation factors, inhibiting coagulation.
In this review inhibitors of coagulation and their effects on cancer progression in *in vitro* and *in vivo* models as well as in patients is discussed.

VTE in cancer

A multitude of cancer subtypes, such as pancreatic and brain cancers, are associated with an increased risk of VTE in patients. The risk of recurrent thrombosis is also increased in cancer patients compared to VTE patients without cancer,[5] and this risk itself is associated with the severity of the cancer; a higher grade confers an increased VTE incidence.[14] Conversely, patients with an unexplained VTE have a significantly increased risk of having an as of yet undiscovered malignancy.[15]

The reported odds-ratios of developing VTE among cancer patients vary from 4.1[2] to 6.7[16], and in the latter study the odds ratio increased to 58.0 when distant metastases were present. This leads to a 4.3% VTE incidence per year in cancer overall, but certain subtypes show a higher VTE incidence, namely pancreatic (11%), brain (8%), lung (4.5%), hematologic (4%) and colorectal cancer (3%).[17] Interestingly, when tumour subtypes are split in high or low grade, the cumulative probability of VTE is doubled in high grade tumours.[18]

As cancer patients have an increased risk of (recurrent) venous thrombosis, patients presenting with a VTE episode receive more specialized treatment. Cancer patients with an acute VTE receiving the LMWH Dalteparin have a 50% lower risk of developing a recurrent VTE, as compared to patients receiving oral anticoagulant treatment, while the rate of major bleeding remained similar.[19] A more recent study showed that patient with advanced or metastatic cancer benefited from ULMWH Semuloparin administration during chemotherapy, reducing the VTE incidence significantly.[20]

Effects of anticoagulants on cancer progression in patients

Besides reducing VTE incidence in cancer patients, there is a large body of evidence that anticoagulant therapy, in the form of LMWH or vitamin K antagonists, influences tumour progression and patient survival (Table 1). In studies as early as 1984 warfarin therapy was associated with prolonged survival in small cell lung carcinoma[7]. A study published in 1992 showed

increased survival in patients given LMWH versus un-fractioned heparin.[21] In a 1999 meta-analysis, LMWH administration resulted in a three month survival benefit over UFH in cancer patients.[22] Importantly, this effect remained consistent in subgroups of specific types of cancer and was not the result of VTE-related deaths.

In a study of cancer patients with advanced solid malignancies but without VTE, the effect of subcutaneous nadroparin administration on patient survival was tested and a prolonged survival was observed.[23] In another study, the use of heparin reduced death by metastatic disease by 50% three years after undergoing surgery for cancer[24]. When dalteparin is administered in combination with chemotherapy in small cell lung cancer patients, tumour response and survival improves significantly.[25]

Regular aspirin use has been associated with a long-term benefit regarding cancer incidence and metastasis, at least for a subset of cancer types. A large meta-analysis by Algra *et al.* shows a decrease in colorectal cancer incidence in long-term aspirin use, with an odds-ratio of 0.62 (95% CI 0.58-0.67, 17 studies combined).[26]

Effects of anticoagulants on patients with a good prognosis

In the FAMOUS study by Kakkar *et al.*, the effect of chronic administration of LMWH in cancer patients without VTE was studied. Dalteparin did not influence the survival of advanced cancer patients, although a subgroup of patients with a relatively good prognosis had a significant survival advantage after two and three years.[27] In a study with cancer patients without metastatic disease, a subcutaneous dose of dalteparin resulted in improved survival compared to oral anticoagulant treatment.[28] A study tracking cancer incidence in a large population based study comparing long-term VKA use to a control group showed a 0.88 hazard ratio of cancer incidence (95% CI 0.8-0.98, $P < 0.015$). When looking at specific types of cancer, only the prostate cancer group showed a significantly reduced hazard ratio when VKA use was compared to control.[29] Non-metastatic prostate adenocarcinoma patients undergoing radiotherapy and using

anticoagulants (warfarin, clopidogrel, and/or aspirin) showed a reduced metastasis rate.[30] These data suggest that long-term anticoagulant treatment might have an influence on early tumour development, because the effects do not appear to impact survival of patients with high-grade or metastatic disease.

Most patient studies investigating cancer and anticoagulant treatment focus on VTE incidence rather than cancer progression as a primary outcome. This has limited the possibilities to study the effects of anticoagulants on tumour progression. A recent meta-analysis by Sanford *at al.* found no significant survival benefit for cancer patients without VTE receiving LMWH.[31] Furthermore, the studies mentioned either use warfarin or LMWH, that may not have an effect on the TF:FVIIa complex. Especially TF:FVIIa has recently been found to influence tumour growth in vitro and in vivo.[32–34] Several drugs targeting this complex are currently under clinical investigation, such as Alt-836[35] and HuMax-TF-ADC,[36,37] both targeting TF in locally advanced or metastatic tumours. It remains to be seen whether these compounds will have clinical benefits, as well as possible adverse effects on haemostasis.

Effects of anticoagulants on tumour development *in vitro* and *in vivo*

A multitude of anticoagulants have been tested *in vitro*, as to whether they can inhibit tumour cell proliferation. The effects of LMWH on tumour cell proliferation have been obscure. In a melanoma study, UFH and LMWH did not influence tumour cell proliferation.[38] In an osteosarcoma cell model, exposure to thrombin increased cell proliferation, and this effect was attenuated when LMWH were added, suggesting that inhibition of thrombin generation using LMWH might be beneficial for attenuating primary tumour growth. Indeed, LMWH was able to suppress primary tumour growth *in vivo* using TF-expressing osteosarcoma cells.[39] In another study, MDA-MB-231 and 4T1 breast cancer cells were exposed to dabigatran but this did not influence cell proliferation or viability.[40] Overall,

these data do not point to an anti-proliferative effect of anticoagulants on tumour cells.

Apart from anti-thrombotics, a multitude of specific inhibitors of coagulation proteins have been studied for their ability to reduce tumour growth both *in vitro* and *in vivo* (Table1). Tissue Factor Pathway Inhibitor (TFPI) is a potent endogenous inhibitor of the extrinsic coagulation pathway by blocking the TF:FVIIa complex via its Kunitz domains. The loss of TFPI-2 correlates with a higher grade in human glioma cell lines and tumour samples[41], and when TFPI-2 expression is restored in a glioma cell line this reduced the size and number of colonies in an *in vitro* colony assay[42]. Furthermore, TFPI-2 inhibited matrigel invasion of glioblastoma cells in a dose-dependent manner.[41]

The thick-derived coagulation inhibitor Ixolaris, which contains two Kunitz-like domains, is able to form a quaternary TF:FVIIa:FXa:Ixolaris complex similar to TFPI[43]. Ixolaris was able to inhibit both TF:FVIIa and TF:FVIIa:FXa complex signaling on breast tumour cells *in vitro*.[44].. *In vivo*, it showed potency to decrease primary tumour growth in human glioblastoma and mouse melanoma models. [45]

rNAPc2, a nematode anticoagulant protein that specifically inhibits TF:FVIIa, significantly suppresses tumour growth in mice in both a lung[33] and colorectal cancer model[46]. Interestingly, the specific FXa inhibitor rNAP5 had no effect on tumour growth[33] suggesting that it is not necessarily coagulation activation that drives tumour growth. And indeed TF signaling has been shown to promote tumour growth independently of it's coagulant function[32]. Therefore direct TF signaling inhibitors might have the most benefit when used to restrain tumour growth.

In vivo tumour growth is limited by both cell proliferation as well as angiogenesis. Activation of the coagulation cascade leads to increased angiogenesis via the protease activated receptors (PARs), i.e. PAR-1 (activated by thrombin and TF:FVIIa:FXa) and PAR-2 (activated by TF:FVIIa and TF:FVIIa:FXa) as shown in Figure 1. PAR-dependent angiogenesis mainly promotes enhanced expression of pro-angiogenic factors by tumour cells[47], activating the surrounding endothelium. The effects of

anticoagulants on *in vivo* tumour growth might thus be effective because they limit angiogenesis rather than cell proliferation.

Non-haemostatic signaling of TF-dependent PAR-2 activation leads to increased mRNA levels of VEGF[48], IL-8 and CXCL-1[32,47] and promotes angiogenesis.[49] PAR-2 activation leads to phosphorylation of the cytoplasmic domain of TF, which activates cell signalling via mitogen-activated protein (MAP) kinases.[50] Blockade of TF or PAR-2 by specific antibodies can effectively attenuate this PAR-2 dependent IL-8 upregulation, tumour growth and density of CD-31^{+} tumour vessels.[32] In MDA-MB-231mfp breast cancer cells this effect can be inhibited using Ixolaris and effects are similar to those observed after PAR-2 inhibition with a blocking antibody.[44] Interestingly, alternatively spliced Tissue Factor (asTF) can also induce angiogenesis, though in a PAR-2 and FVIIa independent manner.[51] AsTF is a soluble, non-coagulant isoform of TF, and is widely expressed in breast[52] and pancreatic[53] cancer. AsTF promotes cell proliferation *in vitro* as well as tumour growth *in vivo*, both of which can be inhibited by a specific antibody[52].

Tissue Factor pathway inhibitor (TFPI) was shown to inhibit endothelial cell formation, as well as blocking phosphorylation of vascular endothelial growth factor (VEGF) receptor 2, suggesting that TFPI can inhibit angiogenesis via its carboxyl terminus.[54]

The LMWH nadroparin was shown to inhibit angiogenesis in a rodent skinfold chamber model.[55] LMWH bemiparin and ultra (U)LMWH RO-14 inhibit angiogenic effects on endothelial cells when exposed to tumour cell-conditioned medium from leukemia, breast, and lung cancer cells in tube formation and migration assays.[56] Another study showed that LMWH affects fibrin matrices *in vitro*, leading to impaired capillary formation using human micro vascular endothelial cells, as well as attenuating their proliferation.[57] LMWHs are able to reduce capillary tube formation, an effect not observed when UFH was used.[58] The phosphorylation of VEGF-mediated KDR (VEGF receptor-2) in human umbilical vein endothelial cells (HUVECs) was diminished after the LMWH fraxiparin administration, as well as vessel formation in an *in vivo* matrigel plug assay.[59]

Inhibition of experimental metastasis by anticoagulants

The rationale behind anticoagulants as possible inhibitors of cancer metastasis is based on evidence that tumors cells benefit from activation of the coagulation cascade: clot formation by tumor cells facilitates attachment to the endothelium[60], clots protect tumor cells from vascular shear stress[61] and facilitates evasion of immune surveillance.[62,63] Furthermore, platelet interaction with tumour cells drives epithelial-mesenchymal transition (EMT) via the TGFβ/SMAD and NF-κB pathways[64], and both platelets and P-selectin promote experimental liver metastasis.[65]

Metastatic spread of tumour cells is the leading cause of mortality, and LMWH shows a limited, but positive effect on cancer patient survival as described above. The effects of heparins on experimental metastasis models has been reviewed in detail by Niers *et al;*[66] in 14 out of 17 reviewed studies heparins reduce either primary or secondary metastasis. A common *in vivo* model for metastasis is the subcutaneous injection of tumour cells such as the B16 melanoma cell line and quantifying lung metastases after a given time period. Several groups have shown that LMWH administration can significantly limit lung seeding of melanoma cells.[38,67] *In vitro,* UFH and LMWH pre-treatment significantly decreased melanoma cell migration and matrigel invasion[38].

Thrombin is capable of enhancing metastasis by platelet activation and fibrin formation, as well as triggering tumour cell signalling via PARs. Activation of coagulation by TF on cancer cells leads to platelet clots around the tumour cells, whereby macrophages are attracted, leading to increased survival of metastatic cells.[68] Platelet aggregation around tumour cells also limits natural killer (NK) cells in targeting the tumour cells.

Besides anticoagulants used for VTE treatment, other specific coagulation inhibitors have been tested for their ability to inhibit metastasis. The Heparin-like heparin sulphate 6-O-sulphotransferase-2 was shown to reduce TGF-β-induced IL-11 expression *in vitro* as well as in *in vivo* tumour progression in bone.[69] In an *in vitro* human umbilical cord vein model, S-

NACH-a modified heparin lacking inhibition of FXa and FIIa-, as well as the LMWH tinzaparin, inhibited adhesion and invasion of MPanc96 human pancreatic tumour cells.[70] S-NACH was even shown to be more potent in reducing surgically induced metastasis compared to tinzaparin, and S-NACH treatment led to reduced E-cadherin expression in pancreatic cancer cells.[71]

Hirudin is a potent thrombin inhibitor naturally occuring in leech saliva. It was shown to diminish B16 melanoma tumour cell induced lung tumours, possibly acting through PAR-1[72]. The potent TF/FVIIa inhibitor rNAPc2 diminishes lung metastases in the B16 melanoma mouse model,[33] and the specific FXa active site inhibitor rAcAP reduced pulmonary metastasis after tail vein injections of LOX human melanoma cells in SCID mice.[73] Furthermore, active site-inhibited FVIIa (FVIIai), which competes with FVII for binding of TF, reduces the number of tumour foci on the lungs of mice injected with B16F0 melanoma cells.[74]

Future directions

The association between increased risk for VTE and a malignant state has been widely established over the last century. Whether treatment of VTE in cancer patients is also beneficial for the treatment of malignancy is less clear. The use of LMWHs only shows increased survival in certain subgroups of patients, namely patients with a better prognosis. Another limitation is the lack of studies in which the effect of anticoagulants on cancer progression is measured as the primary outcome, which limits the conclusions that can be drawn from observed effects of survival of VTE patients with cancer. LMWHs affect the angiogenic behaviour of tumour cells both *in vitro* and *in vivo*, which would confirm the beneficial effects found in patients.

Recently, novel oral anticoagulants (NOACs) have shown great promise in clinical trials, and both direct thrombin (dabigatran) and FXa inhibitors (apixaban and rivaroxaban) are now approved for use in the U.S.[75] As the role of both thrombin and PAR-2 signalling (activated by both the TF:FVIIa

and the TF:FVIIa:FXa complex) have been clearly established as potent contributors to cancer progression we may expect a benefit for cancer patients in curbing both VTE and cancer progression. Recently, the safety and efficacy of NOACs has been described in cancer patient cohorts: use of dabigatran and warfarin resulted in comparable VTE recurrence and bleeding.[76] A meta-analysis showed that dabigatran as well as FXa-inhibitors are effective and safe for use in cancer patients.[77] As the use of NOACs becomes more accepted it will soon become clear whether systematic thrombin or FXa inhibition will also slow cancer progression. Lastly, a striking benefit of blood coagulation research is the identification of non-coagulant peptides and proteins that do show promise in attenuating oncogenic processes. S-NACH, a sulphated non-anticoagulant heparin, showed higher potency in attenuating metastasis in experimental models compared to tinzaparin, a LMWH.[71] This suggests that it is not necessarily the anticoagulant effects of heparins that influence metastasis. Recent publications have moved asTF out of the realm of a non-coagulant and somewhat misunderstood protein to a potent and widely occurring oncogene.[51,53,78] Furthermore, TFs non-haemostatic signalling via PARs is now firmly accepted as a pro-angiogenic pathway.[32,79] Although these insights steer away from blood coagulation, they do provide exciting angles for future research in understanding cancer metastasis.

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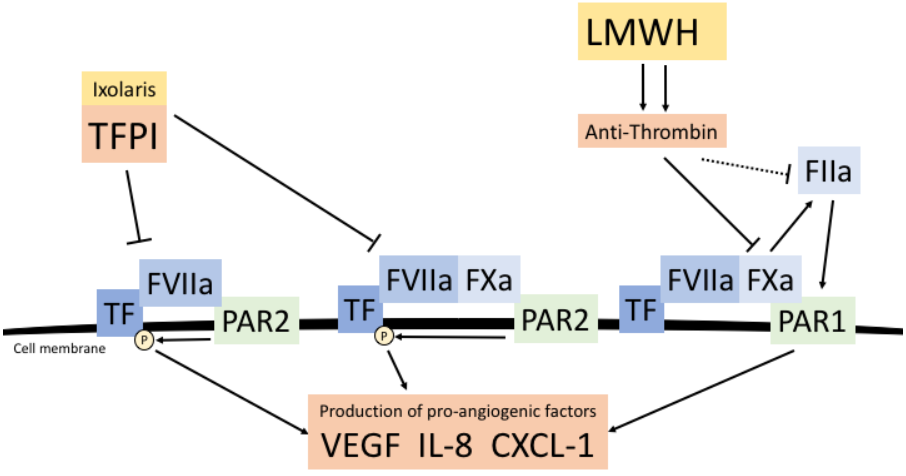

Figure 1. TF:FVIIa signalling promotes angiogenesis. TF:FVIIa and TF:FVIIa:FXa can both activate PAR2. PAR1is activated by TF:FVIIa:FXa as well as FIIa/thrombin. TFPI and ixolaris inhibit the TF:FVIIa and TF:FVIIa:FXa complexes. Anti-thrombin (AT) inhibits both FXa and FIIa, and LMWHs greatly accelerate the ability of AT to inhibit FXa.

Table 1. Overview of studies reporting benefit of anticogulants on cancer progression

	patients	*In vitro*	*In vivo*
	References		
Cancer Incidence			
Aspirin	26		
VKA	29		
Patient Survival			
Warfarin	7		
Heparin	24		
LMWH	21,22,23,25,27,28		
Proliferation/Tumour Growth			
LMWH		39	39
TFPI-2		42	
Ixolaris			45
rNAPc2			46
TF antibody			32
asTF antibody		52	52
Dabigatran			40
Angiogenesis			
LMWH		56,57,58,59	55,59
TFPI		54	
Ixolaris		44	
TF antibody		32	32
Metastasis			
Warfarin	30		
clopidogrel	30		
aspirin	30		
UFH	66	38	
LMWH	66	38,70	38,67
TFPI-2		41	
6-O-sulphotransferase-2		69	69
S-NACH		70,71	71
Hirudin			72
rNAPc2			33
rAcAP			73
FVIIai			74
Dabigatran			40

Chapter 3 – Alternatively spliced Tissue Factor promotes breast cancer growth in a β1 integrin-dependent manner

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Summary

Full-length tissue factor (flTF), the coagulation initiator, is overexpressed in breast cancer (BrCa), but associations between flTF expression and clinical outcome remain controversial. It is currently not known whether the soluble alternatively spliced TF form (asTF) is expressed in BrCa and/or impacts BrCa progression. We report for the first time that asTF, but not flTF, strongly associates with both tumor size and grade, and induces BrCa cell proliferation by binding to 1 integrins. asTF promotes oncogenic gene expression, anchorage-independent growth, and strongly upregulates tumor expansion in a luminal BrCa model. In basal BrCa cells that constitutively express both TF isoforms, asTF blockade reduces tumor growth and proliferation *in vivo*. We propose that asTF plays a major role in BrCa progression acting as an autocrine factor that promotes tumor progression. Targeting asTF may comprise a novel therapeutic strategy in BrCa that stems tumor growth, yet does not impair normal hemostasis.

Introduction

In breast cancer (BrCa), proteins that modulate splicing events such as ASF/SF2 and SR(Serine/Arginine-rich)p55, are frequently upregulated and contribute to cell transformation (1,2). BrCa cells exhibit specific alternative splicing signatures that were proposed as potential prognostic factors in BrCa (3). Alternative splicing of proteins such as spleen tyrosine kinase (Syk), p53, PTEN, CXCR3 and Rac1 impacts BrCa cell behavior and therefore, disease progression (4-8).

Full-length Tissue Factor (flTF) is the initiator of blood coagulation (9). Following vascular damage, flTF binds its ligand FVII(a) which triggers clot formation. Aside from sub-endothelial tissues, flTF is also abundant on cancer cells (9) and fuels tumor progression by modulating integrin 3 1 function, cell migration (10), and FVIIa-dependent Protease Activated Receptor (PAR)2 activation, while flTF- 1 integrin complexation enhances PAR2 activation (11). flTF-dependent PAR2 activation results in the

production of VEGF, CXCL1 and IL-8, thus promoting the angiogenic switch and consequently, tumor growth *in vivo* (10,11).
Alternative splicing of TF pre-mRNA results in the deletion of exon 5 and thus a frameshift in exon 6, yielding a transmembrane domain-lacking isofom that can be secreted (12). Human and murine alternatively spliced TF (asTF) contain novel C-termini with poor homology to one another or any other protein, and the murine asTF C-terminus is longer than that of human asTF. (12,13). High expression of asTF in tumor cell lines suggests a role in tumor progression (10,14). Subcutaneous growth of pancreatic cancer cells overexpressing asTF results in larger and more vascularized tumors (15). We recently discovered that asTF induces angiogenesis, independent of PAR2 activation, by acting as an integrin ligand (16). Thus, flTF and asTF facilitate cellular signaling via distinct mechanisms critical to tumor cell behavior.
Currently, nothing is known about asTF expression and function in breast cancer. Regulated splicing of TF pre-mRNA in human monocytes is controlled by several SR proteins, including ASF/SF2 and SRp55 (17,18). Expression of SR proteins is frequently perturbed in BrCa tumors (2), and it is thus plausible that the relative abundance of flTF and asTF is also altered in BrCa.
Prior studies that attempted to correlate "TF expression" in BrCa specimens with clinical parameters such as tumor grade and disease outcome did not discriminate between flTF and asTF (19,20). Thus, there is uncertainly as to how the two TF isoforms associate with/contribute to BrCa progression. We set out to characterize flTF and asTF expression in a large set of BrCa tissue specimens. Identified associations were tested *in vitro* using innovative cell models, and *in vivo* with recapitulation of distinct BrCa subtypes.

Results

asTF expression positively correlates with breast cancer grade and stage.

Prior studies pointed to a role for TF in tumor progression (19-21). To explore whether asTF and flTF differentially contribute to BrCa progression, we analyzed asTF and flTF protein expression in a BrCa tissue array (TA) comprising specimens from 574 BrCa patients (22). asTF and flTF were detectable in >95% of the BrCa specimens with various degree of tumor cell positivity. Healthy mammary tissue showed limited expression of asTF compared to flTF (asTF: 4% of all specimens, flTF: 38%; Fig. S1*A*). Specificity of previously validated flTF- and asTF-specific antibodies (23) was re-confirmed (Fig. S1*B*). Confocal analysis revealed flTF localization on the cell membrane, while asTF was mostly intracellular (Fig. S1*C*).
Expression of asTF positively correlated with histological grade as well as tumor size (Table I), while flTF expression only correlated with grade. asTF expression also correlated with age. No significant associations were found between asTF or flTF levels and estrogen receptor (ER), progesterone receptor (PgR), or human epidermal growth factor receptor 2 (HER2) status. This raises the possibility that asTF impacts BrCa progression in a manner qualitatively distinct from that of flTF.

asTF expression enhances proliferation of BrCa cells.

To explore the mechanistic link between the unique correlation of asTF expression and BrCa clinical parameters, we constructed MCF-7 cells harboring a genomic FRT insertion, thus containing a unique locus-specific DNA acceptor site and established several FRT lines, selecting clone 2A3-3 for further studies. Insertion of asTF and flTF cDNA resulted in similar mRNA expression levels, but intracellular protein levels of asTF were lower (Fig. S2*A,B*), likely due to asTF secretion (see below and Fig. 1*G*). Confocal analysis revealed that flTF was localized on the plasma membrane, confirmed by FACS analysis (Fig. S2*D*), while asTF was present in vesicular structures (Fig. 1*A*). Only 2A3-3-flTF cells exhibited significant coagulant activity (Fig. S2*C*). asTF protein levels were ~5 fold higher in 2A3-3-asTF cell lysates than in lysates of tumor TA specimens with high asTF positivity, likely due to the constitutive asTF overexpression of 2A3-3 cells and the appreciable presence of asTF-negative stroma (up to 50%) in our tumor

specimens (Fig. S2*E*). 2A3-3 cells expressing flTF, asTF, an aspecific protein (-Galactosidase), or empty vector control cells (pcDNA) were tested in an MTT proliferation assay and compared with parental MCF-7 cells. 2A3-3-pcDNA and 2A3-3- Gal proliferation rates were identical to those of MCF-7 (Fig. 1*B*), but proliferation rates of 2A3-3-asTF cells were increased by ≥2-fold, while those of 2A3-3-flTF cells were only modestly increased. Cell counting and genomic DNA measurements confirmed these results (Fig. S3*A*,*B*). An independently established second 2A3-3-asTF line showed similar proliferation rates (Fig. S3*C*) and TF-specific shRNA eliminated enhanced proliferation (Fig. 1*C*,*D*), confirming that the effect was asTF-dependent. Because asTF contains an unusual C-terminus due to a frameshift, a possibility exists that asTF expression may increase cell proliferation through activation of the unfolded protein response (UPR). However, we found no evidence for UPR activation and/or protein aggregation neither in flTF- nor asTF-expressing cells (Fig. S3*D*,*E*) Increased cell numbers were also not due to increased cell survival, as all cell lines exhibited similar viability levels (Fig. S3*F*). asTF and flTF expression resulted in down-regulation of cell cycle inhibitors and enhanced phosphorylation of the pro-mitogenic p42/p44 MAP kinase (Fig. 1*E*). Effects of flTF and asTF expression on proliferation were somewhat less pronounced in an MCF-7 FRT clone (2A1-2) with lower flTF/asTF expression (Fig. 1*F*,*G*, Fig. S3*G*). 2A1-2 cells exhibited decreased asTF secretion, while intracellular asTF levels were equal to those in 2A3-3 cells (Fig. 1*G*), suggesting that asTF secretion is important to the enhancement of proliferation. Indeed, co-culture of control cells with asTF-expressing cells increased the proliferation of control cells (Fig. 1*H*). Culturing control cells in 2A3-3-asTF-conditioned medium enhanced proliferation, and asTF depletion from the medium reversed this effect (Fig. S4*A*). Addition of recombinant asTF to pcDNA cells increased proliferation at concentrations as low as 1 nM, well below asTF concentrations detectable in the plasma of metastatic breast cancer patients (Fig. S4*B*,*C*). Incubation of asTF-expressing 2A3-3 cells with an asTF-blocking antibody, but not with an flTF-blocking antibody, reduced asTF-dependent cell proliferation (Fig. 1*I*). Limited flTF-

elicited proliferation was not dependent on PAR2, as incubation with PAR2- and FVII-blocking antibodies was without effect (Fig. S4*D*). These results demonstrate that secreted asTF enhances BrCa cell proliferation in an autocrine fashion.

asTF augments pro-oncogenic gene expression.

We compared gene expression profiles in asTF-expressing 2A3-3 cells with those in 2A3-3-pcDNA or flTF expressing 2A3-3 cells. Compared to 2A3-3-pcDNA or flTF expressing cells, asTF expression upregulated genes involved in cell cycle progression (e.g. *CCNA1*), tumor proliferation (e.g. *MDK*), cytoskeletal reorganization/motility (e.g. *FERMT2*), invasion (e.g. *FAM5c*), and cell survival (e.g. *MSLN*) (Fig. 2*A*,*B*). Moreover, expression of asTF downregulated several tumor suppressors (e.g. *CDH18*), and genes involved in cell cycle arrest (e.g. *EFEMP1*) and apoptosis (*DRAM1*) (Fig. 2*A*,*B*, Table SI). Expression of SRPK2 that activates the TF pre-mRNA splicing regulator ASF/SF2 (24) was altered by asTF and flTF, suggesting that TF splice variants regulate their own expression. These results indicate that asTF enhances BrCa cell proliferation via modulation of cell cycle regulators, proliferation inducers, and tumor suppressors/pro-apoptotic proteins.

asTF enhances proliferation by binding to β1 integrins.

Because asTF induces angiogenesis via binding integrins on endothelial cells (EC) (16), we reasoned that asTF-dependent BrCa cell proliferation may also be integrin-dependent. Silencing of the 1 integrin subunit resulted in diminished proliferation of 2A3-3-asTF cells (Fig 3*A*,Fig S5*A*) and flTF cells (Fig. S5*A*,*B*), but not 2A3-3-pcDNA cells; shRNA silencing of the 3 integrin subunit, which is not expressed in these cells, was without effect (Fig. S5*C*).

Artificially truncated recombinant flTF ("sTF") was recently found to induce EC proliferation by binding to the integrin 1 region between amino acid (aa) residues 579 and 799 (25). Because sTF contains the entire N-

terminal region of asTF, we tested whether asTF binds to this integrin region. A 1 aa 579-799 domain-specific antibody and a peptide mimicking this domain inhibited proliferation of 2A3-3-asTF, but not that of control cells or 2A3-3-flTF cells, even after prolonged incubation (Fig. S5*D*,S5*E*). Co-immunoprecipitation (IP) demonstrated that asTF directly binds to 1 integrin and binding was lost after pre-incubation with the 1 aa 579-799 antibody (Fig 3*B*). Binding of asTF to 1 integrins was confirmed using a modified ELISA, showing that asTF binds well to recombinant 6 1, (Fig. S5*F*), which is consistent with our earlier findings (16). Pre-incubation of control cells in suspension with recombinant asTF enhanced cell binding to collagen and fibronectin, but not vitronectin (Fig. S5*G*), indicating that asTF binding may modulate integrin activation; functional blockade of 1 reversed this effect (Fig. 3*C*). In support of an activating effect of asTF on 1 integrins, reactivity of HUTS-21, an antibody that recognizes the active conformation of 1 integrins, was increased when 2A3-3 cells expressed asTF or were exposed to recombinant asTF (Fig. 3*D*). To ascertain asTF's co-localization with 1 integrins, we pre-incubated pcDNA cells with fluorescently-tagged asTF; partial co-localization with 1 integrins on the cell surface was observed (Fig. S5*H*). As 1 integrin blockade in 2A3-3 cells only partially inhibited asTF-dependent proliferation, these results suggest that, aside from 1 integrins, asTF likely binds to other membrane-associated proteins.

asTF induces anchorage-independence and tumor growth in vivo.

We next investigated the effects of asTF expression on oncogenic potential using soft-agar assays. While asTF did not affect the number of colonies, it caused a 3-fold increase in colony size; the impact of flTF expression on colony size was marginal (Fig. 4*A*,*B*).

asTF-dependent tumor growth was then assessed orthotopically. asTF expression increased tumor expansion (Fig. 4*C*), while flTF-expressing 2A3-3 cells yielded tumors that were similar in size or smaller than those formed by control cells. asTF-expressing cells yielded large tumors with little

stroma, whereas control and flTF cells gave rise to small tumor islands surrounded by stroma (Fig. S6). flTF and asTF protein expression was confirmed in tumors *in vivo*, ruling out that poor growth of flTF-expressing cells was due to loss of flTF expression (Fig. S6). asTF-expressing tumors had more CD31+ capillaries and macrophage infiltrate, and contained more proliferating tumor cells, specifically at the tumor periphery (Fig. 4*D*,*E*). These data demonstrate that asTF promotes BrCa cell proliferation *in vitro* and *in vivo*.

asTF blockade reduces growth of BrCa cells expressing endogenous asTF.

While we dissected the role of asTF in tumor growth using MCF-7 cells constructed to express either asTF or flTF, native asTF is co-expressed with flTF. Moreover, MCF-7 cells express low levels of PAR2, which may mechanistically explain the lack of 2A3-3-flTF-dependent tumor expansion *in vivo*. Because MDA-MB-231 BrCa cells express high levels of flTF and PAR2 (11), we employed them to assess the role of asTF in an flTF/PAR2-positive setting. Although asTF levels in MDA-MB-231 cells were low (Fig. 5*A*), a more aggressive MDA-MB-231 sub-line that had been isolated from the mammary fat pad following orthotopic implantation (MDA-MB-231-mfp) (26) had significantly higher asTF levels, while flTF levels were unchanged. In agreement, spliceosomal proteins that promote biosynthesis of asTF mRNA were upregulated in MDA-MB-231-mfp cells (Fig. S7*A*). Importantly, asTF levels in these cells were lower or equal to the asTF levels in BrCa specimens (Fig S2*E*). asTF-specific antibody blockade significantly inhibited proliferation of MDA-MB-231-mfp cells (Fig. 5*B*), but had no effect on proliferation of the parental MDA-MB-231 line, demonstrating functional specificity. Selective anti-flTF antibody 10H10 did not inhibit proliferation of either cell type *in vitro*, which is consistent with the notion that flTF potentiates angiogenesis – but not proliferation – in these cells (11) (Fig. 5*B*). A 1 integrin-blocking antibody and/or the aa 579-799 integrin peptide also inhibited proliferation of MDA-MB-231-mfp cells, but did not further reduce proliferation in the presence of the anti-asTF antibody (Fig.

5*C*), confirming that asTF augments proliferation in MDA-MB-231-mfp via 1 integrins. 3 integrin blockade was without effect (Fig. 5*D*).
We then implanted MDA-MB-231-mfp cells orthotopically in NOD-SCID gamma (NSG) mice in the presence or absence of asTF-blocking antibody. asTF blockade with as little as 100 g antibody significantly inhibited tumor growth (Fig. 5*E*) and resulted in a reduction of the proliferation zone at the tumor periphery (Fig. 5*F*, Fig. S7*B*). Notably, asTF blockade did not reduce vascular density, suggesting that asTF does not impact angiogenesis in a model featuring a pro-angiogenic flTF-PAR2 axis (Fig. 5*G*, Fig. S7*B*). Thus, asTF blockade decreases the tumorigenic potential of BrCa cells expressing native asTF, flTF, and PAR2.

Discussion

Until now, contributions of asTF to tumor progression have remained unclear. This is the first study that reports i) asTF's selective abundance in BrCa tissue, ii) asTF-dependent, autocrine augmentation ofBrCa cell proliferation, and iii) the efficacy of anti-asTF monoclonal antibodies in stemming BrCa growth. Analysis of BrCa specimens from 574 patients revealed that asTF positively correlates with both grade and the T-status of cancer lesions as well as the patients' age, while flTF correlates solely with tumor grade, and is detectable in ~40% of normal breast tissue, compared to ~4% for asTF. We here demonstrate that asTF upregulates BrCa cell proliferation irrespective of its impact on angiogenesis in an asTF overexpression model, as well as in BrCa cells that express native asTF. In contrast to asTF-triggered proliferation, flTF-triggered proliferation rates were low in our TF overexpressing cells; further, flTF-dependent proliferation was not observed in an aggressive MDA-MB-231 cell line that expresses asTF. Thus, asTF appears to be the major TF variant that promotes BrCa cell proliferation .
asTF upregulated genes that play pivotal roles in cell cycle progression and proliferation. *CNNA1* and *CNNA2*, important regulators of cyclin-dependent kinases during S phase, and *ANAPC10* were significantly upregulated in 2A3-3-asTF cells compared to control or flTF expressing

cells. Growth factors (*MDK, GAL* and *TIMP1)* were also upregulated. Although we observed upregulation of some cell survival genes, we found no evidence for altered cell survival in asTF-expressing cells. Still, it cannot be ruled out that these genes contribute to the cumulative impact of asTF expression on tumor xenografts.

Our studies further revealed that asTF-integrin interactions were responsible for increased proliferation of BrCa cells. asTF co-localized with and bound to 1 integrins and 1 integrin silencing reversed asTF-dependent proliferation. An antibody against the 1 region encompassing residues 579-799 or a peptide mimicking this domain, reversed asTF-dependent – but not flTF-dependent – proliferation, indicating that asTF binds to this distinct 1 region. We hypothesize that asTF induces a conformational change in 1 integrins that render them prone to activation, as we used a 1 integrin-blocking antibody that is reactive with the membrane-proximal -tail domain (TD) of the 1 integrin subunit, and the 579-799 integrin peptide features this domain. The TD contains a CD-loop that contacts the ligand-binding integrin A domain and the hybrid domain, and this contact is lost upon integrin activation (27,28). It has been postulated that the CD-loop acts as a deadbolt, preventing integrin activation by locking A in an inactive state. Indeed, a number of antibodies that activate 1 integrins bind to the TD or the A interface, and the TD has been shown to regulate ligand binding (27,29). We propose that asTF induces the removal of the CD-loop deadbolt. However, direct conformational effects of asTF on 1 integrin function may not be the sole means by which asTF modulates the "integrin profile" of BrCa cells: asTF expression also upregulates FERMT2, a positive regulator of integrin activation (30), and suppresses TSN3, a negative regulator of integrin function (31).

The *in vitro* phenotype of asTF-expressing 2A3-3 cells (enhanced proliferation and soft agar colony growth), was recapitulated *in vivo* while flTF-expressing cells that proliferated only moderately faster than control cells *in vitro*, produced tumors of the same size as control cells. It is not clear why flTF overexpression in MCF7 cells did not enhance tumor growth *in*

vivo, but the paucity of PAR2 expression may be a contributing factor. PAR2 is instrumental in flTF-dependent tumor angiogenesis (11), and poor expansion of these cells may result from a lack of PAR2-dependent angiogenesis. This is in agreement with the results of *in vivo* experiments employing PAR2-expressing MDA-MB-231-mfp cells: asTF blockade did not affect vascular development in tumor xenografts, although we did not directly test the influence of asTF-induced angiogenesis on BrCa growth. Our results indicate that asTF does not significantly influence angiogenesis in the MDA-MB-231-mfp xenograft model, while upregulation of CD31+ vessels in asTF-expressing 2A3-3 tumors suggests that asTF-dependent angiogenesis may be a contributing factor in 2A3-3 xenografts. Differences in secreted asTF levels and/or presence of a functional flTF-PAR2 axis in MDA-MB-231-mfp cells may explain why asTF differently affects vascular density in these two xenograft models.

Alternative splicing has been deemed critical to the proliferation of BrCa cells: overexpression of ASF/SF2, a major SR protein and regulator of the flTF/asTF mRNA ratio in monocytes (18), leads to enhanced proliferation, transformation, and BrCa growth *in vivo* (1). Further, the expression of SRp40 – the spliceosomal protein that promotes asTF synthesis (17), whose levels are upregulated in MDA-MB-231-mfp cells – is increased in human BrCa, and associates with lymph node metastasis (32). It may be of interest to investigate whether the effects of heightened SRp40 expression in BrCa are in part dependent on asTF production.

In conclusion, autocrine asTF expression induces integrin-mediated BrCa cell proliferation that contributes to tumor growth, rendering asTF a novel target for anti-cancer strategies that modulate the biological activity of this minimally coagulant TF form, thereby avoiding adverse impacts on hemostasis.

Experimental Procedures

Reagents, cell culture, proliferation assays, western blotting, microarray analysis and soft agar experiments.

See supplemental information.

Immunofluorescence studies.

For HUTS-21 staining, cells were fixed in methanol for 5 minutes. In all other experiments, cells were fixed in 2% formaldehyde, permeabilized with 0.1% Triton-X100 when appropriate. Cells were incubated O/N with primary antibodies followed by incubation with secondary antibodies conjugated to Alexa-488 or Alexa-594. Coverslips were mounted using Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). In some experiments, cells were incubated with fluorescently-conjugated asTF for 20 min, before fixation. Images were acquired using a Leica SP5 confocal microscope and a Leica DMI6000B.

Orthotopic breast cancer models and immunohistochemistry.

Animal experiments were approved by the animal welfare committee of the Leiden University Medical Center. 5 animals per experimental group were used. 2A3-3 cells ($2*10^6$ in 50 l) were injected into the inguinal fat pad of NOD-SCID mice (Charles River, Maastricht, The Netherlands). Tumor growth was measured using calipers and the formula Volume=(length*width*width)/2. For MDA-MB-231-mfp growth *in vivo*, $0.5*10^6$ cells were injected in fat pads of NOD-SCID-gamma mice (Charles River, Maastricht, The Netherlands). After completion of the experiment, mice were sacrificed and tumors were extracted and fixed in 4% formalin. Sections were de-paraffinized, rehydrated, endogenous peroxidase activity was blocked with 0.3% H_2O_2. Antigen retrieval was done in sodium citrate buffer for 10 min at 100˚C. Sections were blocked with 10% normal goat serum in PBS and incubated overnight at 4˚C with primary antibody.

Sections were incubated for 30 min with Envision (Dako, Heverlee, Belgium), visualized using DAB, and counterstained with hematoxylin.

Tissue microarray analysis.

A tissue array containing tumor material from 574 non-metastasized breast cancer patients that mostly underwent tumor resection at Leiden University Medical Center (LUMC) between 1985 and 1994 (22). Approval was obtained from the LUMC Medical Ethics Committee. Age, tumor grade, histological type, TNM status, median follow-up (17.9 years), locoregional or distant tumor recurrence, and expression of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) were known. Tumors were graded according to the current pathological standards. Normal mammary tissue of 266 patients (46%) was available for analysis. Sections were cut and stained for flTF and asTF as described above. The percentage of asTF and flTF positive tumor cells was scored by two blinded observers. Patients in the 1st quartile were deemed negative.

Statistical analyses.

Assessment of the associations between flTF/asTF expression and clinical variables were performed using SPSS and Stata. Cohen's kappa coefficient for inter-observer agreement was 0.85 and 0.88 for asTF and flTF, respectively. The χ^2 test was used to evaluate associations between clinicopathological parameters and asTF/flTF expression. Analysis of *in vitro* and *in vivo* experiments was carried out using t-tests. Mean and stdev are shown in figures, unless stated otherwise. Significant differences in bar graphs are indicated by * ($p<0.05$), ** (p,0.01) and *** (p,0.001).

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Author contributions

BK, YWvdB, CT, designed and performed experiments, analyzed data and wrote the paper. JSDM, EdK, CE analyzed data. MAvdE performed experiments. PJK, WR, CJvdV and GJL provided essential material and reagents. PHR and SO provided essential feedback and wrote the paper. VYB and HHV wrote the paper, supervised the studies, and prepared the final draft.

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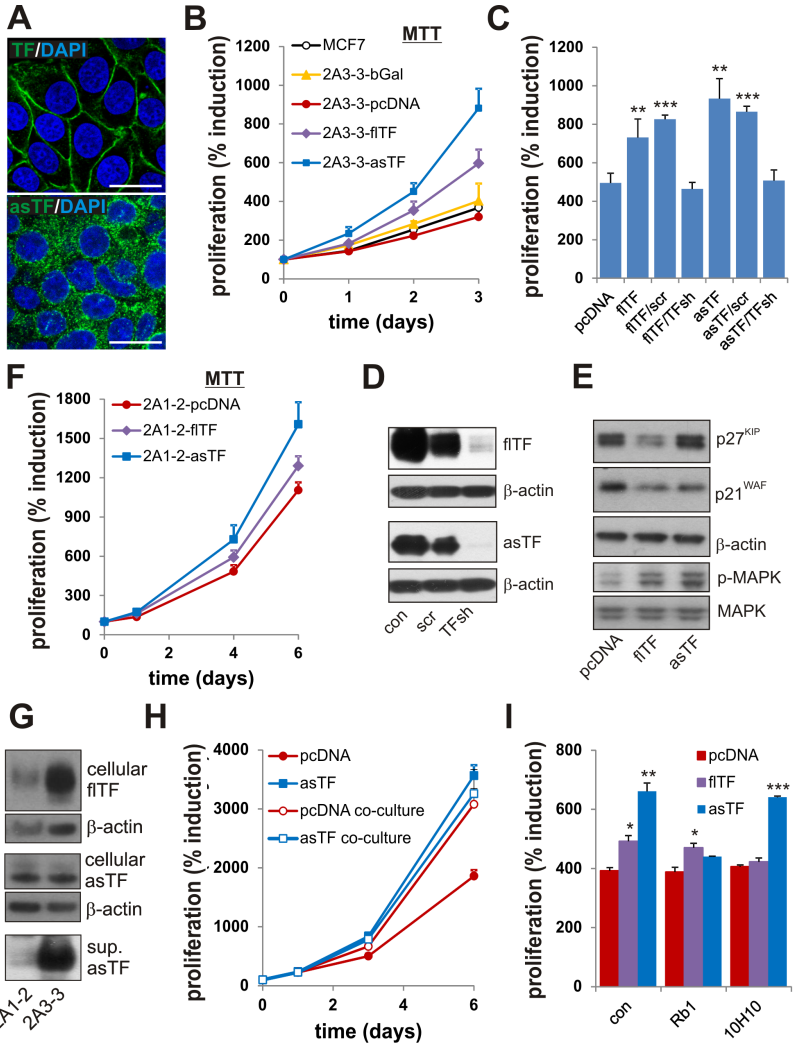

Fig. 1. asTF expression induces cancer cell proliferation. (*A*) FRT cells (clone 2A3-3) received flTF or asTF cDNA by homologous recombination. Localization of flTF (upper panel) and asTF (lower panel)) was assessed by confocal microscopy using specific antibodies (monoclonal flTF-specific Ab 4509 and polyclonal asTF-specific Ab, as described before (23)). Scale bars: 25 m. (*B*) Proliferation of 2A3-3 cells containing the cDNAs indicated was monitored using MTT assay. (*C*) 2A3-3-flTF or 2A3-3-asTF were transduced with TF-specific or control shRNA. Cells were subjected to MTT assay 3 days after the start of the experiment. (*D*) flTF and asTF expression in 2A3-3 cells transduced with TF-specific or control shRNA constructs. (*E*) Expression of cell cycle inhibitors and MAP kinase phosphorylation was assessed in 2A3-3 cell lines. (*F*) Cell line (2A1-2) harboring an FRT site at a transcriptionally less active region received empty vector, flTF or asTF cDNA and proliferation was monitored using MTT assay. (*G*) Cell-associated and secreted levels of asTF and flTF in 2A3-3 and

2A1-2 cells. (*H*) pcDNA cells and asTF-expressing cells were grown separately, or in adjoining 12-well plates containing an open port, allowing asTF diffusion to control cells. Proliferation was monitored using MTT assay. (*I*) 2A3-3-pcDNA, 2A3-3-flTF, or 2A3-3-asTF cells were treated with 50 g/ml flTF-signaling blocking antibody (10H10) or asTF-blocking antibody (RabMab1, Rb1), proliferation was assessed 3 days later using MTT assay.

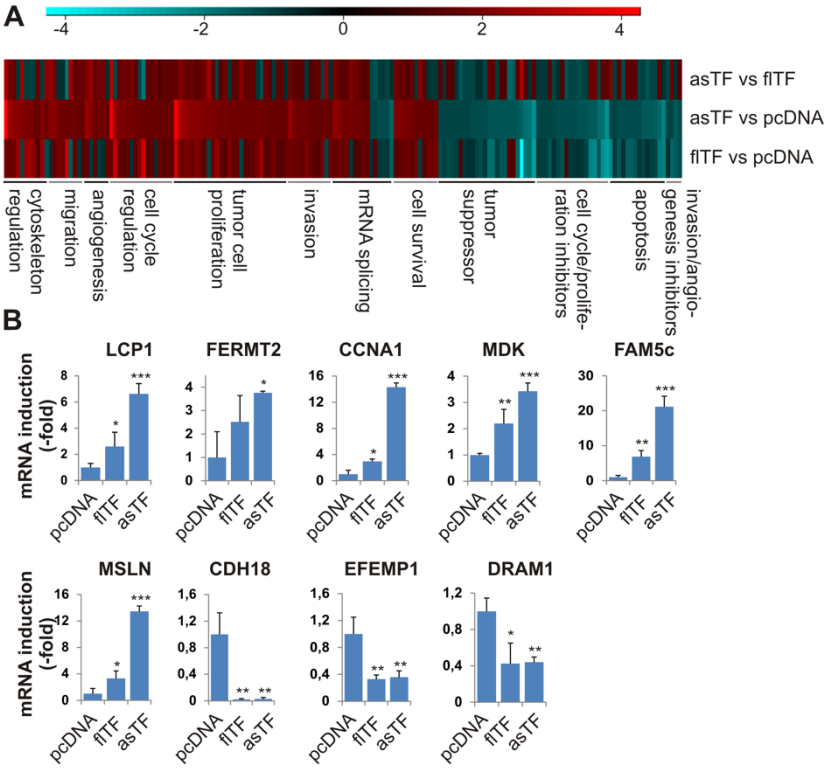

Fig. 2. Microarray analysis of flTF and asTF expressing cells. (*A*) Heat map representation of relevant genes in flTF and asTF expressing 2A3-3 cells, compared to control. The heat map comprises the genes whose expression was significantly up- or downregulated (P<0.05) by at least 1.33-fold. Results of 4 independent experiments are shown; red and blue denote up- and downregulation, respectively. (*B*) Upregulation of selected genes was verified using real-time PCR.

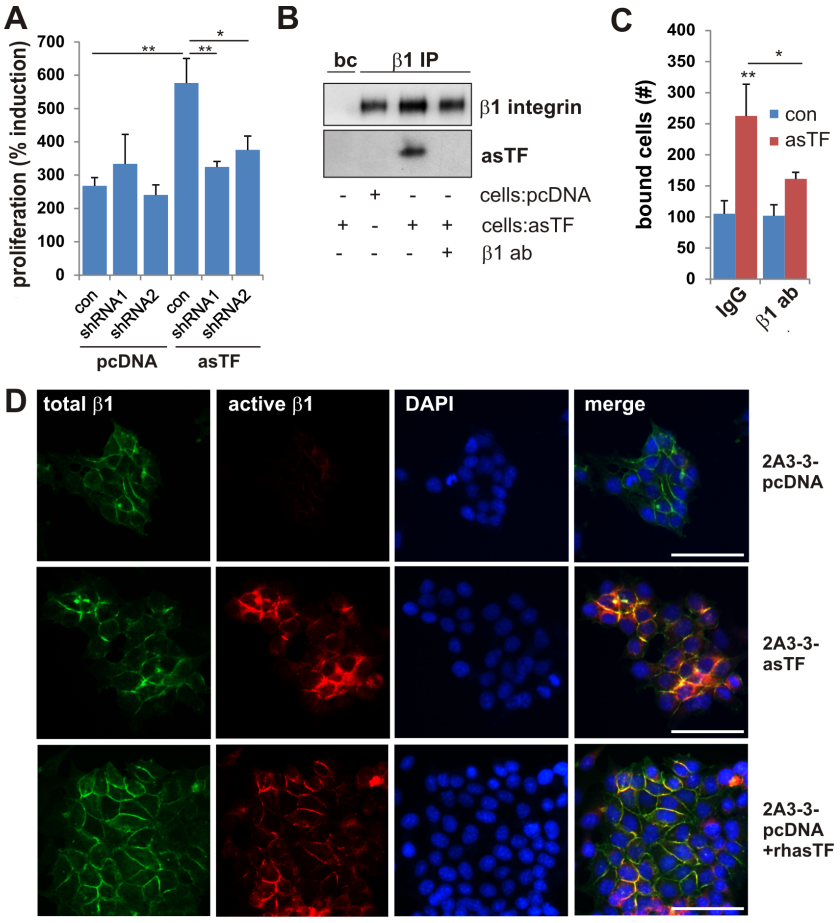

Fig. 3. TF variant-induced proliferation is integrin dependent. (*A*) pcDNA control cells or asTF-expressing 2A3-3 cells were transduced with shRNA to silence 1 integrins, and proliferation of these cells was compared to control after 3 days. (*B*) 2A3-3-pcDNA or –asTF cells were mock treated or incubated with a 1 (epitope 579-799)-reactive antibody. The cells were lysed in Brij78-containing lysis buffer and 1 integrin was precipitated using AIIB2. asTF co-immunoprecipitation was assessed by Western Blot. (*C*) 2A3-3-pcDNA cells were preincubated with 10 nM recombinant asTF in the presence or absence of an antibody against 1 integrin epitope 579-799 and seeded on collagen I. After washing, remaining cells were counted. (*D*) 2A3-3-asTF cells or 2A3-3-pcDNA cells, untreated or incubated with 10 nM recombinant asTF, were fixed and stained with AIIB2 (total 1 integrin; green) and HUTS-21 (activated 1 integrin; red). Scale bars: 50 m.

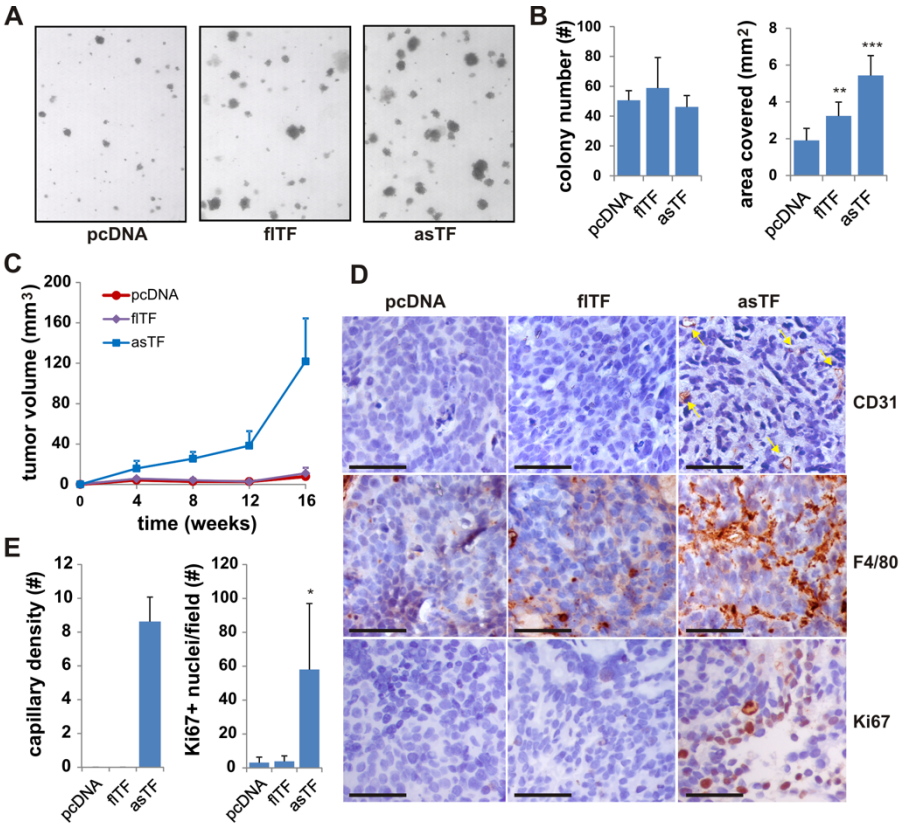

Fig. 4. TF variant-induced transformation and tumor growth. (*A*) 2A3-3-pcDNA, flTF or asTF cells were seeded in soft agar and allowed to grow for 14 days. Images were captured and colony number per area covered determined using ImageJ (*B*). (*C*) 2A3-3-pcDNA, flTF or asTF cells were injected into the mammary fat pad of NOD-SCID mice, and tumor growth assessed for 16 weeks. Mean and SEM are shown. (*D*) Tumors were analyzed by immunohistochemistry to assess vascular density (CD31), macrophage infiltration (Mac3), and proliferation rate (Ki67). Scale bars: 50 m. (*E*) Quantification of vascular structures per view field.

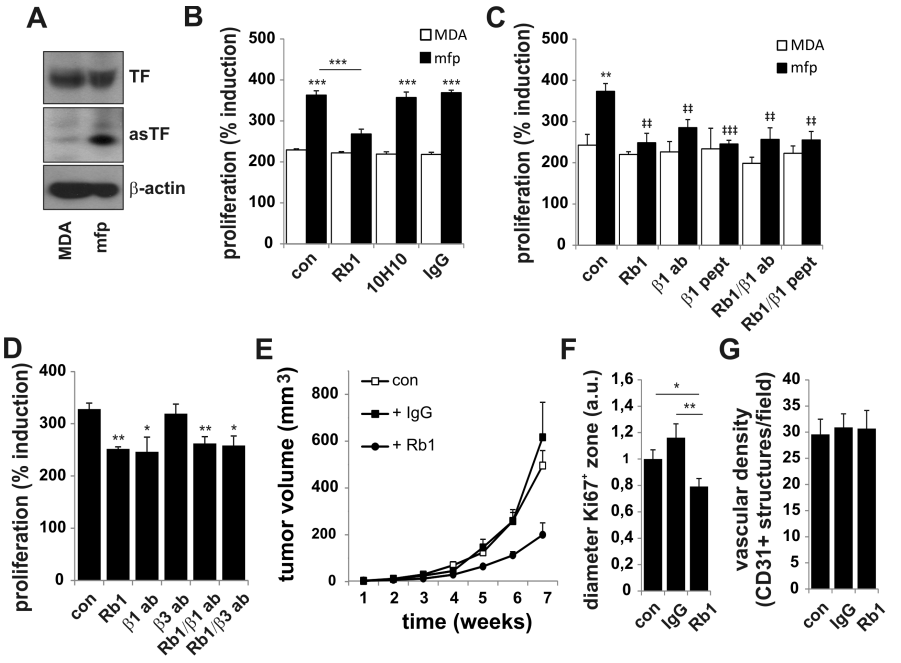

Fig. 5. asTF induces tumor cell proliferation of BrCa cells endogenously expressing flTF/asTF/PAR2. (*A*) flTF and asTF expression in MDA-MB-231 and MDA-MB-231-mfp cells. -actin is used as a loading control. (*B*) MDA-MB-231 (MDA) and MDA-MB-231-mfp (mfp) cells were grown in the presence of asTF-specific antibody (Rb1), flTF-specific antibody 10H10, or IgG control. Proliferation was assessed after 3 days using MTT assay. (*C*) As in B, but blocking antibodies against 1 integrins or the 1 peptide was added separately or in combination with Rb1. * indicates significant differences compared to control (MDA-MB-231) cells, ‡ indicates significant differences compared to MDA-MB-231-mfp cells (*D*) As in B, a 3-blocking antibody was added. (*E*) MDA-MB-231-mfp cells were injected in the mammary fat pad of NOD-SCID-gamma (NSG) mice in the presence or absence of 100 g Rb1 or IgG control. Mean tumor volume and SEM are shown. (*F*) Tumor specimens were stained with anti-Ki67, and the proliferation zone diameter (Ki67+ area at the periphery of the tumor) was determined. Values are expressed as arbitrary units, with the control set at 1. (*G*) Quantification of CD31-positive structures in the tumor specimens shown in *F*.

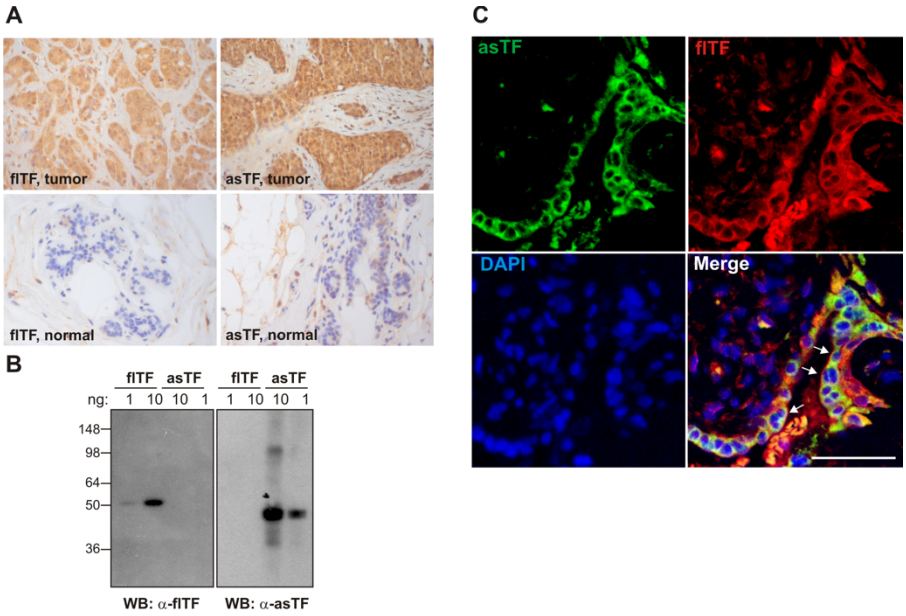

Fig S1

Fig S1. asTF and flTF are overexpressed in human breast cancer specimens. (*A*) Representative photographs of tissue microarray punches of human breast cancer specimens (upper panels) and matched normal mammary tissue (lower panels) immunohistochemically stained using an flTF-specific (left panels) or an asTF-specific antibody (right panels). Brown color indicates positive staining. (*B*) Specificity of the anti-flTF and anti-asTF antibodies, tested on Western Blot. The indicated amounts of purified flTF and asTF proteins were loaded on gel. Note the asTF dimers migrating at 98 kD. (*C*) Representative confocal image of a breast cancer specimen double stained with the antibodies in (*A*). Note the intracellular staining of asTF (green) and the flTF staining at the cell periphery (red). Examples of peripheral staining are indicated by arrows.

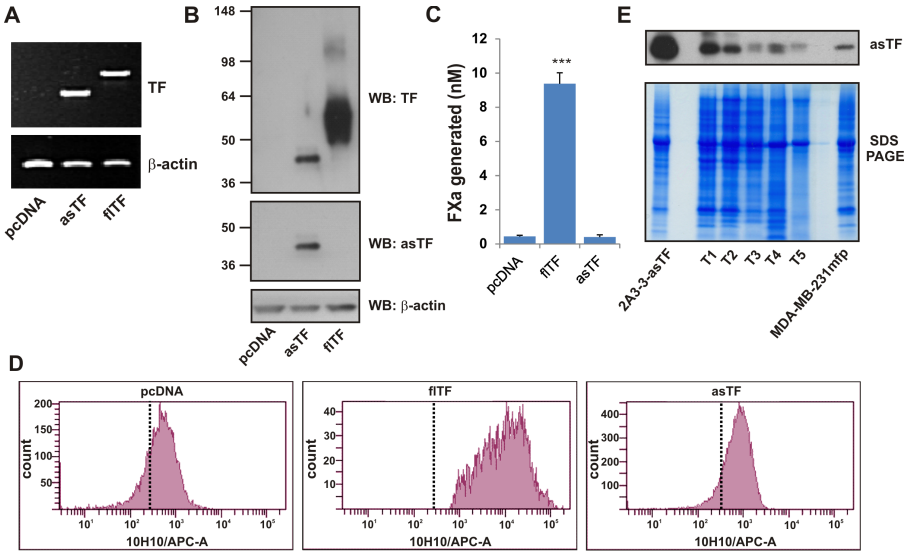

Fig S2

Fig S2. Characterization of TF isoform-expressing 2A3-3 cells. (*A*) Control vector (pcDNA), flTF or asTF cDNA were stably integrated into the FRT site of 2A3-3 cells and TF isoform expression was verified by PCR using TF-specific primers. Primers against -actin were used as a control. (*B*) Expression of flTF and asTF in 2A3-3 cells assessed by Western Blot. Antibodies specific for total TF and asTF (monoclonal rabbit antibody RabMab1) were used. (*C*) TF activity of 2A3-3 control cells and cells expressing flTF or asTF was measured after addition of 10 nM FVIIa and 100 nM FX in HBS. FXa generation was determined after 30 minutes using a Spectrozyme FXa kinetic assay. (*D*) FACS analysis of 2A3-3 control cells and cells expressing flTF or asTF. Cells were labeled in suspension with 1 g/ml 10H10 and APC-labeled rabbit-anti-mouse secondary antibody. Labeled cells were analyzed on a Becton Dickinson LSR II. Dashed lines indicate Mean Fluorescence Intensity of the same cells, labeled with IgG control. (*E*) 50 ug protein from lysates prepared from 2A3-3-asTF, MDA-MB-231mfp cells and 5 tumor specimens with high asTF expression (as determined in the tissue array analysis) were subjected to Western Blotting using anti-asTF RabMab1 as the primary antibody. The same samples were also loaded on SDS-PAGE and stained using Coomassie to verify equal loading.

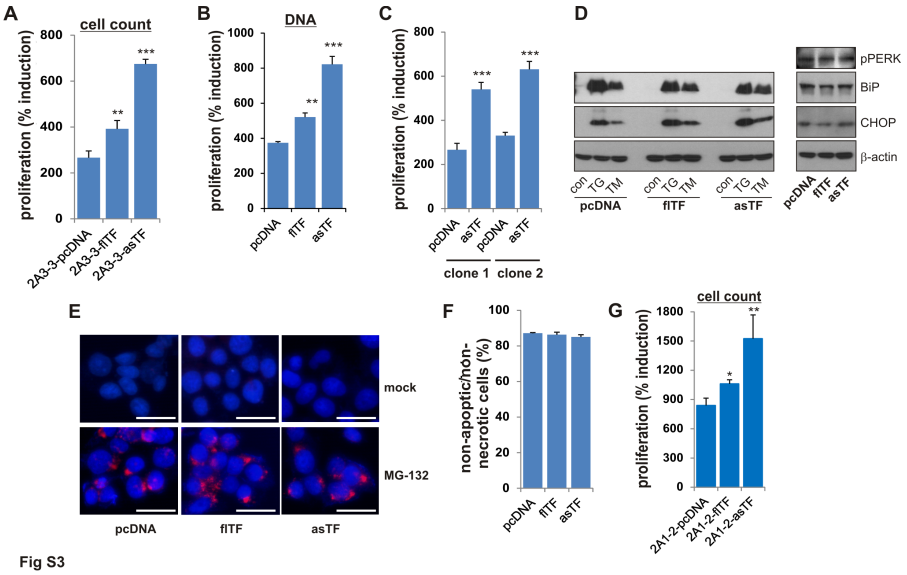

Fig S3

Fig. S3. Proliferation of asTF-expressing 2A3-3 cells. (*A*) Proliferation of 2A3-3 cells expressing TF variants was determined using cell counts. (*B*) Proliferation of 2A3-3 cells expressing TF variants was determined using DNA contents of cellular lysates at day 0 and day 3. Proliferation was expressed as % increase compared to day 0. (*C*) Enhanced proliferation of an independently generated second asTF clone was determined using MTT assays. (*D*) Activation of the Unfolded Protein Response (UPR), as determined by Western Blotting. The indicated cell lines were screened for upregulation of UPR-responsive proteins CHOP and BiP, and phosphorylation of PERK. -actin was used as a loading control. The left panel shows a short exposure of CHOP and BiP levels in these cells, using established UPR activators (2 M Thapsigargin (TG) and 10 g/ml Tunicamycin (TM)) as positive controls. The right panel shows basal levels (long exposure) of CHOP and BiP, and PERK phosphorylation in these cells. (*E*) Presence of cellular protein aggregates was determined using the ProteoStat protein aggregation kit (Enzo, Farmingdale, NY, USA). The proteasome inhibitor MG-132 was used as an inducer of protein aggregation (positive control). Images were acquired with a conventional fluorescence microscope (Leica DMI6000B). Scale bars represent 20 m. (*F*) Apoptosis was assessed using Annexin V-FITC staining and subsequent FACS analysis. (*G*) A cell line (2A1-2) containing an FRT site at a transcriptionally less active region was equipped with an empty vector, flTF or asTF cDNA by homologous recombination, and proliferation was monitored using cell counting.

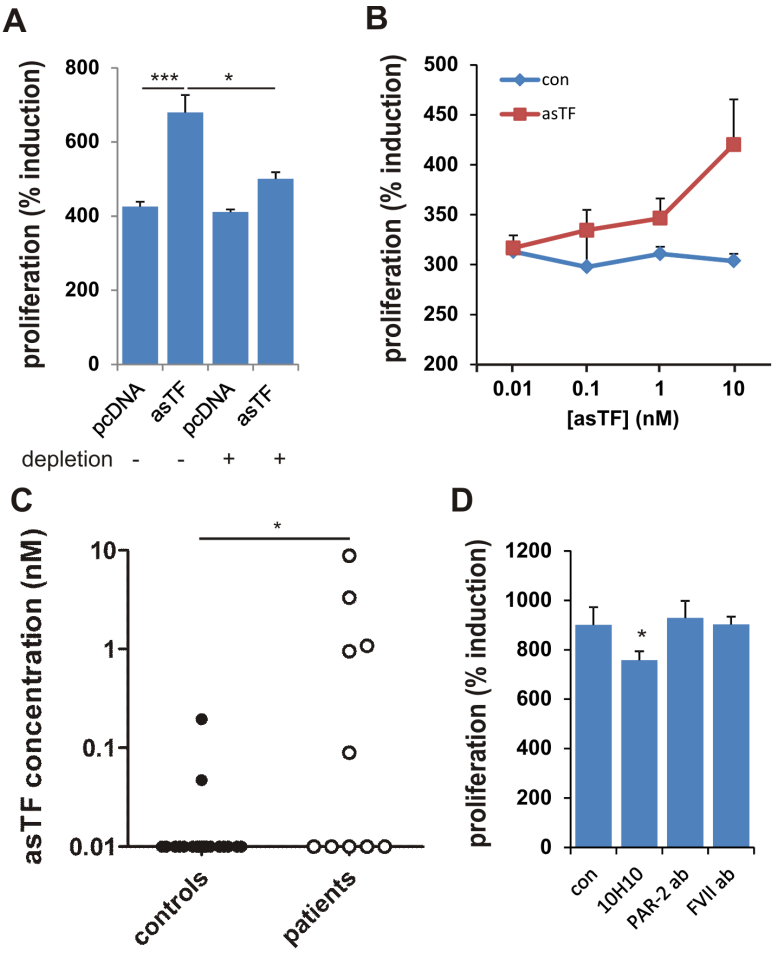

Fig S4

Fig. S4. Proliferation of 2A3-3-asTF cells is dependent on asTF secretion. (*A*) Conditioned medium of 2A3-3-asTF cells was left untreated or asTF-depleted using an asTF-specific antibody RabMab1. 2A3-3-pcDNA cells were cultured in these medium preparations, and proliferation was assessed 3 days later using MTT assay. (*B*) Effects of indicated amounts of recombinant asTF on 2A3-3-pcDNA cell proliferation using MTT. The experiment was controlled (con) by adding the identical corresponding amounts of the vehicle. (*C*) asTF concentrations were determined in plasma from 20 healthy control subjects and 10 metastatic breast cancer patients using an asTF-specific ELISA. (*D*) flTF cells were incubated with 50 g/ml 10H10, PAR-2 blocking antibody, or FVII-blocking antibody. Increases in cell number were determined on day 4, using MTT assay.

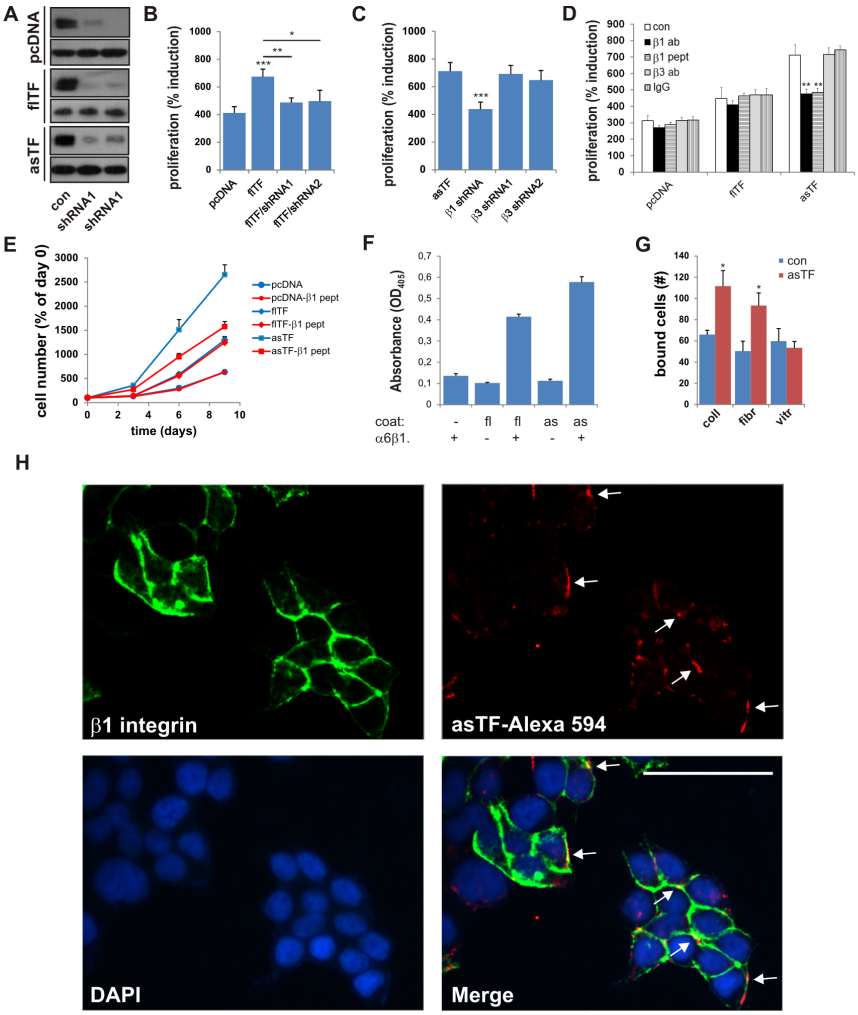

Fig S5

Fig. S5. Effect of integrin blockade on flTF- or asTF-expressing 2A3-3 proliferation. (*A*) 2A3-3-pcDNA, -flTF, and -asTF cells were transduced with 1 integrin shRNA lentiviral particles. The Western Blot shows 1 integrin expression levels after lentiviral transduction, -actin is used as a loading control. (*B*) Proliferation of 1-silenced 2A3-3-flTF cells was assessed after 3 days using MTT assay and compared to that of control cells (pcDNA). (*C*) Cells were transduced with 1 integrin-specific shRNA particles or 3 integrin-specific shRNA particles as a control. Cell proliferation was determined by means of MTT assay. (*D*) 2A3-3 cells were incubated with antibodies against 1 integrins (residues 579-799), 3 integrins, or a peptide representing integrin 1 residues 579-799. (*E*) Proliferation was assessed after the days indicated as described. 2A3-3-pcDNA cells (circles), -flTF cells (diamonds) or -asTF cells (squares) were incubated with a 1 peptide resembling the 1 integrin region between residues 579-799 (1 nM). Proliferation was followed as in *A*. (*F*) 96 wells plates were

coated with asTF. Truncated flTF (sTF) coating was included as a positive control, as flTF was previously shown to bind to 6 1 integrins. Subsequently, the plates were incubated with the indicated recombinant integrin dimers, and bound integrin was detected using 1 antibody TS2/16 HRP-conjugated anti-mouse secondary antibody and colorimetric substrate (TMB). (*G*) 2A3-3-pcDNA cells were pre-incubated with 10 nM recombinant asTF and seeded on collagen I, fibronectin, or vitronectin pre-coated dishes (all 10 g/ml) and left to adhere for 60 min in HEPES-Tyrode buffer. After washing, the remaining cells were counted. (*H*) pcDNA cells were grown on coverslips and incubated with 10 nM recombinant asTF conjugated to Alexa 594 for 20 minutes at ambient temperature. After fixation, cells were labeled with 1 antibody AIIB2 (green). Arrows indicate areas of extensive co-localization. Scale bar: 50μm.

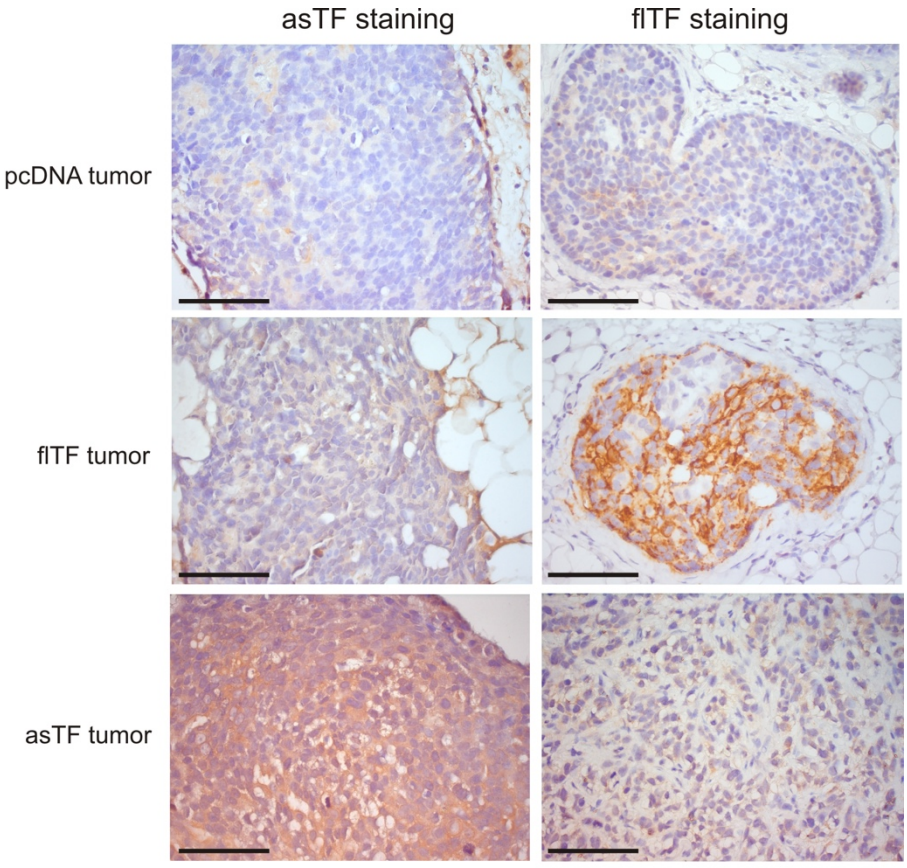

Fig S6

Fig. S6. Morphology and TF variant staining of 2A3-3 tumors. 2A3-3-pcDNA, -flTF or -asTF-derived tumors were extracted, fixed and stained using specific antibodies for asTF (left panels) or flTF (right panels). Sections were counterstained with hematoxylin. Note differences in flTF and asTF staining patterns and tumor morphology of pcDNA and flTF tumors versus asTF tumors. Scale bars: 100 μm.

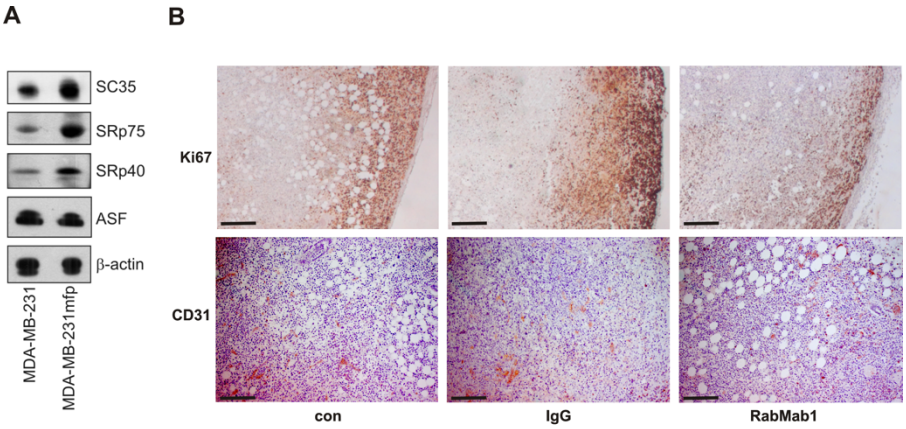

Fig. S7. Characteristics of MDA-MB-231-mfp tumor cells and MDA-MB-231-mfp-derived tumors. (*A*) Expression of SC35, SRp75 and ASF/SF2 were determined on Western Blot. β-actin was determined as loading control. (*B*) MDA-MB-231-mfp cells were co-injected with buffer control (con), 100 μg IgG control antibody or 100 μg RabMab1. Tumors were extracted, fixed in formalin, and stained for Ki67 (5x magnification) or CD31 (10x magnification). Notice the presence of mammary fat cells in the tumor specimens (white). Scale bars represent 200 μm.

Chapter 4 – Alternatively spliced Tissue Factor synergizes with the estrogen receptor pathway in promoting breast cancer progression

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Abstract

BACKGROUND: Pro-coagulant full length Tissue Factor (flTF) and its minimally coagulant alternatively spliced isoform (asTF), promote breast cancer (BrCa) progression via different mechanisms. We previously showed that flTF and asTF are expressed by BrCa cells, resulting in autoregulation in a cancer milieu. BrCa cells often express hormone receptors such as the estrogen receptor (ER), leading to the formation of hormone-regulated cell populations.
OBJECTIVE: To investigate whether TF isoform-specific and ER-dependent pathways interact in BrCa.
METHODS: TF isoform-regulated gene sets were assessed using ingenuity pathway analysis (IPA). Tissues from a cohort of BrCa patients were divided into ER positive and ER negative groups. Associations between TF isoform levels and tumor characteristics were analyzed in these groups. BrCa cells expressing TF isoforms were assessed for proliferation, migration and *in vivo* growth in the presence or absence of estradiol.
RESULTS: IPA analysis pointed to similarities between ER- and TF-induced gene expression profiles. In BrCa tissue specimens, asTF expression associated with grade and stage in ER positive but not in ER negative tumors. flTF only associated with grade in ER positive tumors. In MCF-7 cells, asTF accelerated proliferation in the presence of estradiol in a 1 integrin-dependent manner. No synergy between asTF and the ER pathway was observed in a migration assay. Estradiol accelerated the growth of asTF-expressing tumors but not control tumors *in vivo* in an orthotopic setting.
CONCLUSION: TF isoform and estrogen signalling share downstream targets in BrCa; concomitant presence of asTF and estrogen signalling is required to promote BrCa cell proliferation.

Key words: blood coagulation, integrin beta1, cell movement, cell proliferation, tumors

Introduction

Breast cancer (BrCa) is the most widespread cancer type among women. The World Health Organization (WHO) has estimated 508,000 breast cancer-related deaths in 2011, placing BrCa in the group of solid cancer forms with the highest mortality rates. Seventy percent of all BrCa tumors are estrogen receptor (ER) positive [1, 2]. ER exists in two isoforms, ERα and ERβ, but the effects of estrogen are mainly regulated by the former. Interestingly, ERα's expression levels are higher in the tumor compartment compared to healthy tissue [3]. ER resides in the cytoplasm in complex with HSP90. Upon estradiol (E2) binding, HSP90 dissociates from the complex and ER dimers translocate to the nucleus where ER binds to Estrogen Responsive Elements (EREs) located in the promoter regions of target genes [4, 5]. E2-ER-DNA interaction leads to the recruitment of co-activators with histone acetyl transferase (HAT) activity, resulting in chromatin opening and increased gene transcription [6]. Target gene expression plays a major role in proliferation, survival (cyclins D,A,E) [7] and metastasis (MMP2) [8] which are crucial processes in cancer progression [9]. Therefore, anti-cancer treatment based on inhibition of ER signalling is a commonly used strategy. BrCa patients with ERα positivity receive treatments either to block the receptor, or to downregulate estrogen levels, which prolongs recurrence-free survival [10].

Full Length Tissue Factor (flTF) is a 47 kDa transmembrane glycoprotein that initiates blood coagulation [11]. In addition to its clotting function, flTF binding to its ligand, coagulation factor VII/VIIa (FVIIa), leads to the activation of a subset of G-protein coupled receptors termed Protease Activated Receptors (PARs) [12]. In murine models, flTF signalling via PARs is an important contributor to BrCa progression [13], and higher flTF expression levels in tumors associates with poor prognosis [14, 15].

TF pre-mRNA can undergo alternative splicing, yielding a soluble protein termed alternatively spliced tissue factor (asTF) [16]. Although asTF is detected in human thrombi and can, at high concentrations, shorten clotting

times in the presence of negatively charged phospholipids [16], some groups failed to detect asTF procoagulant activity [17, 18]. There is compelling evidence that asTF promotes cancer progression in ways that do not require proteolysis. asTF does not seem to signal via PARs [19]; rather, it facilitates cancer progression via integrin ligation. MiaPaCa-2 pancreatic cancer cells [20] and MCF-7 BrCa cells engineered to synthesize asTF [21] gain a proliferative advantage due to asTF expression, leading to the formation of larger and more vascularized tumors. In contrast, flTF expression did not impact proliferation of MCF-7 cells and, somewhat surprisingly, it severely suppressed *in vivo* growth of MiaPaCa-2 cells, the reasons for which were not determined [20]. In addition, asTF has pro-angiogenic capacity: it ligates $\alpha v\beta 3$ integrin resulting in endothelial cell migration, and $\alpha 6\beta 1$ resulting in capillary formation [19]. Integrin ligation was also shown to be crucial for BrCa cell proliferation, revealing that asTF-integrin signalling is a novel key player in BrCa progression [21]. Contributions of ER and asTF/flTF signalling to BrCa progression render both pathways an attractive therapeutic target. Moreover, blocking both pathways simultaneously may lead to a more pronounced tumor regression. We deemed it of importance to evaluate whether the asTF and ER signalling pathways interact with each other, e.g. by sharing downstream signalling components. At present, studies examining potential flTF/asTF and ER synergy are lacking. Using bioinformatics and a large set of BrCa tumor specimens, we investigated whether TF isoforms and ER pathways are likely to interact. We further confirmed the identified potential associations using a panel of *in vitro* and *in vivo* assays.

Materials and Methods

Ingenuity pathway analysis

flTF and asTF dependent gene expression profiles were determined by microarray analysis as described elsewhere [21]. The top 400 upregulated and downregulated genes were uploaded into the Ingenuity Pathway Analysis application (Ingenuity® Systems, www.ingenuity.com). The gene

set was compared with the profiles in the Ingenuity Pathway Knowledge Base. Associations of asTF, flTF-dependent gene regulation with disease states, cellular functions, and upstream modulators were determined. Fisher's exact test was used to calculate p values.

Tissue microarray analysis

The use of a tissue microarray was approved by the Leiden University Medical Center (LUMC) medical ethics committee. Non-metastasized BrCa samples from 574 patients that underwent surgery at LUMC from 1985 to 1994 were used [21, 22]. Subjects' age, tumor grade, histological type, stage, nodal involvement, PgR, Her2 and ER status were available for analysis. Tissues were stained with flTF and asTF-specific antibodies as detailed before [23, 13]. The percentage of flTF or asTF positive tumor cells was determined and the lowest quartile was deemed negative. X^2 statistical tests were used to evaluate associations between flTF / asTF expression and histopathological characteristics in ER+ and ER- tumors.

Cell culture and viral transductions

FRT site-positive MCF-7 cells (clone 2A3-3) and 2A3-3 cells stably transfected with flTF cDNA, asTF cDNA, or a control vector were described before [21]. All cells were cultured in DMEM (GE Healthcare, Buckinghamshire, UK) with 10% fetal calf serum (FCS), 2mM L-glutamine and Penicillin/Streptomycin. To deplete estrogens, 2 weeks before the experiment the growth medium was switched to phenol red free DMEM (Life Technologies, Carlsbad, CA, USA) with 10% charcoal-stripped FCS (Sigma-Aldrich, St Louis, MO, USA), 2mM L-glutamine, and Penicillin/Streptomycin. Scrambled and β1 integrin-specific shRNA lentiviral particles were generated using shRNA vectors obtained from the Mission Library (Sigma-Aldrich). Transduced cells were selected with 2 μg/ml puromycin.

Proliferation and migration assays

Cellular proliferation rates were determined using MTT assays as described before [21]. In brief, $2x10^4$ cells per well were seeded in 12 well plates and cultured in phenol red free medium containing 10% charcoal stripped FCS. Because of the short half-life of E2, medium was supplemented with 1 nM, 10 nM E2 (Sigma-Aldrich) or ethanol solvent on days 0, 3, and 6. The day after seeding (day 0) and on day 6, cells were incubated with 0.5mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) diluted in PBS, for 30 min at 37°C. Subsequently, MTT-containing PBS was removed and replaced by isopropanol/0.04 N HCl. The solution was transferred to a 96 well plate and OD_{562} was determined. Proliferation rates were expressed as percent increase in the signal compared to day 0. In some experiments, cells were cultured in phenol red-containing media in the presence or absence of the ER antagonist ICI-182,780 (Sigma-Aldrich, St Louis, MO).

Cell migration was assessed using silicone inserts (Ibidi, Martinsried, Germany). Cells were seeded to confluence and the following day, cultures were treated with 12.5 μg/ml mitomycin C (Sigma-Aldrich) for 3 hours to prevent proliferation. Silicone inserts were removed and gap closure was assessed at regular time intervals. The gap area was calculated using Image J software, and migration was expressed as percent closure of the area at the start of the assay.

qPCR

Total RNA was isolated using TRIzol (Life Technologies) and converted to cDNA using the Super Script II kit (Life Technologies). Expression levels of ERα were determined by real-time PCR, using CCA CCA ACC AGT GCA CCA TT as a forward primer and GGT CTT TTC GTA TCC CAC CTT TC as a reverse primer.

Western Blotting

Cells were lysed in 2x sample buffer (Life Technologies, Blieswijk, Netherlands) and samples were denatured at 95°C for 10 min, loaded on

12% polyacrylamide gels (Life Technologies) and transferred to PVDF membranes. Membranes were blocked 1 hr at 4°C and probed with primary and corresponding HRP-conjugated secondary antibodies. Blots were developed using Western Lightning (PerkinElmer, Waltham, MA), and chemiluminescent bands were visualized with X-ray film. Anti-ER antibody was from Cell Signaling (Beverly MA), anti- -actin was from Abcam (Cambridge, UK) and anti-β1 integrin was from Millipore (Amsterdam, Netherlands)

Orthotopic breast cancer model and immunohistochemistry

Animal experiments were approved by the LUMC animal welfare committee. Orthotopic injections (5 animals per group) were performed as described elsewhere [21]. In brief, $2x10^6$ control (pcDNA) or asTF-expressing cells were injected into inguinal fat pads of NOD-SCID mice (Charles River, Wilmington, MA, USA). Simultaneously, estrogen pellets (1.5mg/pellet, Innovative Research of America, Sarasota, FL, USA) were placed subcutaneously. Tumor dimensions were measured with calipers and tumor volume was derived using the formula tumor volume = (length x width x width)/2. Tumors were excised and fixed in 10% formalin solution O/N followed by embedding into paraffin. Sections were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked with 0.3% H_2O_2. Antigen retrieval was performed in EDTA buffer for 10 min at 100°C. Sections were then blocked with 5% Bovine Serum Albumin in PBS and incubated overnight at room temperature with anti-Ki67 (Dako, Glostrup, Denmark) and anti-vWF primary antibodies (Dako, Glostrup, Denmark). Sections were incubated for 1 hr with Envision (Dako, Glostrup, Denmark), visualized using DAB, and counterstained with hematoxylin.

Results

asTF and flTF pathways are strongly homologous to the ER pathway

Our previous work showed that asTF facilitates BrCa expansion [21]. Gene expression analysis using mRNA of control (pcDNA) and asTF-expressing 2A3-3 cells revealed that asTF upregulates the expression of genes affecting proliferation, survival, and invasion; conversely, tumor suppressors and apoptotic genes were downregulated [21].

flTF expression in BrCa cells conferred a moderate proliferative advantage *in vitro* but did not result in increased tumor expansion *in vivo* [21]. To gain a better insight into TF's role in BrCa progression, TF's interaction with other pathways, and to enable a side-by-side comparison of the effects exerted by each TF isoform, we used the microarray data set described before [21] to perform Ingenuity Pathway Analysis (IPA). To explore the possible interacting signalling networks, we uploaded the top 400 upregulated and downregulated genes into the IPA application. The profile of asTF-expressing cells was consistent with the role of asTF signalling in disease states such as cancer (Table 1), which is in agreement with previous findings [20, 21, 24]. Furthermore, asTF expression associated significantly with neurological disorders, organismal abnormalities, and diseases of the reproductive system. The cellular functions impacted by asTF signalling span proliferation, motility, and cell cycle regulation, which again emphasize that asTF plays a role in tumorigenesis (Table 2). Apart from associations with cancerous diseases, flTF-related disorders included neurological and dermatological disorders (Table 1). Furthermore, flTF-dependent gene expression profiles associated significantly with cell proliferation, survival, morphology, and assembly (Table 2), all processes linked to tumor progression.

To investigate how asTF and flTF signalling is regulated in BrCa cells, we used IPA to carry out an upstream regulator analysis. Interestingly, the

asTF-dependent gene expression profile implied strong similarities with ER- and, to a lesser extent, HER2-dependent gene regulation (Supplementary Table 1); we note that ER and HER2 are two major determining factors in BrCa progression. 110 genes showed expression profiles consistent with E2 stimulation, whereas 50 genes are commonly regulated both in asTF and HER-2 dependent signalling. In addition, the asTF pathway also featured common elements of trans-hydroxytamoxifen treatment, a strong ER antagonist [25].
In contrast to asTF, flTF-dependent gene regulation appeared to be linked to the p53 pathway. Myc expression and progesterone are important contributors to BrCa progression [26]. Both of these pathways showed an effect on flTF signalling. flTF-dependent gene expression also showed similarities with estrogen-induced signalling, but less significantly when compared to asTF-induced signalling (Supplementary Table 2). These results suggest that distinct regulators contribute to TF isoform dependent signalling.

asTF expression associates with BrCa grade and stage in ER+ tumors

The partial overlap between asTF and ER signalling pathways prompted us to investigate this link in BrCa tissue specimens [21, 22]. We previously reported significant associations between asTF expression levels and clinical parameters such as histological grade and tumor stage [21]. Of note, asTF expression did not show association with the ER status [21]. We divided the tumor specimens into ER- and ER+ groups and re-evaluated the associations between asTF expression levels and said clinical parameters. Higher asTF expression positively associated with higher T stage and a more advanced histological grade in ER+ tumors (Fig. 1A,B, supplementary table 3), but not in ER- tumors (Fig. 1C,D, supplementary table 3). There was no association between asTF expression and age, histological type, nodal involvement, and PgR / Her2 status in ER+ and/or ER- specimens (supplementary table 3). Analogous analyses performed for flTF revealed the positive association between flTF expression and grade, both in ER+ and

ER- tumor specimens (Fig. 1, supplementary table 4). Interestingly, flTF expression did not associate with stage in ER+ and/or ER- specimens (Fig. 1, supplementary table 4). These findings suggest that asTF, but not flTF, cooperates with the ER signalling pathway to promote BrCa progression.

Estradiol increases the proliferation rate of ER+ BrCa cells in a TF-dependent manner

We have previously shown that asTF-expressing cells exhibit higher proliferation rates in phenol red (PR)-containing media [21]. PR is known to act as a weak ER agonist [32]. Indeed, prolonged incubation with the ER antagonist ICI-182,780 reduced proliferation of all our cell lines to a similar extent (Fig. 2A). Therefore, we assessed proliferation rates of control (pcDNA), asTF- and flTF-expressing cells in phenol red-deficient media. asTF- and flTF-expressing cells did not exhibit proliferative advantage over control cells. Interestingly, treatment of these cells with E2 (1nM, 10nM) enhanced growth rates of flTF and asTF-expressing cells (Fig. 2B,C). Importantly, flTF-expressing cells required higher doses of E2 to show maximal proliferation rate compared to asTF-expressing cells, suggesting that asTF and, to a lesser extent, flTF sensitizes cells to E2. This phenomenon was not due to altered expression of ERα, the sole ER isoform in MCF-7 cells [27], as control, flTF- and asTF-expressing cells had very similar expression levels of ERα (Fig. 2D,E).

Because asTF ligates β1 integrins to promote BrCa proliferation [21], we lowered β1 protein levels in asTF-expressing cells using shRNA (Fig. 2F) and assessed the resultant effects. E2-dependent proliferation was diminished upon β1 downregulation (Fig. 2G), which points to a role for the β1 integrin subset in the observed asTF/E2 synergy. Finally, asTF blockade using our previously characterized asTF-specific monoclonal antibody Rb1 [21] inhibited proliferation in E2 treated cells to a larger extent than that of E2 untreated cells (Fig. 2H). The magnitude of the E2's effect on TF-expressing cells suggests that E2- dependent proliferation is highly dependent on TF function and β1 integrin.

Estrogen and asTF induce migration of BrCa cells

Because asTF is a stronger facilitator of E2-dependent proliferation compared to flTF in our next set of experiments we focused on the asTF isoform. We evaluated the interaction of ER and asTF signaling in BrCa cell migration, a process crucial for metastatic spread. In a modified gap closure assay we observed that, in the absence of E2, asTF-expressing cells migrated faster than pcDNA cells. While the presence of E2 further increased migration rates of pcDNA and asTF cells, we did not observe synergistic effects of asTF expression and E2 stimulation (Fig. 3). These results indicate that, in contrast to cell proliferation, asTF and E2 do not synergize to increase cell migration, yet their effects are additive in potentiating it.

Estrogen increases *in vivo* tumor growth in BrCa cells expressing asTF

We sought to corroborate the functional significance of the asTF/ER synergy *in vivo*. As previously reported, asTF-expressing BrCa cells orthotopically injected into mammary fat pads of mice produce larger tumors than control (pcDNA) cells [21]. To assess the impact of estrogens on BrCa cells *in vivo*, we performed a side-by side comparison of xenografted asTF-expressing and control (pcDNA) cells following subcutaneous placement of estrogen pellets. In line with our previous findings, asTF-expressing cells formed larger tumors compared to control cells [21] and estrogen augmented this effect; remarkably, the volume of tumors formed by control cells did not significantly increase in response to estrogen treatment (Fig. 4A,B). We also evaluated tumor growth rates by Ki67 staining: Ki67 positivity significantly correlated with tumor volume (Fig. 4C,D). Finally, we assessed vascular density in the tumors and observed that the combination of E2 and asTF dramatically increased the levels of vWF-positive structures in tumor tissue (Fig. 4E,F). These findings confirm that estrogen and asTF signalling pathways synergize in BrCa cells *in vivo*.

Discussion

Here, we analysed the synergy between ER- and TF-triggered pathways in BrCa progression. While flTF- and asTF-dependent pathways converge with ER-dependent pathways, we show that asTF is the main isoform synergizing with the ER pathway in BrCa. We base these conclusions on the following observations: i) asTF expression associated with tumor grade and size only in the specimens of ER+ tumors; ii) BrCa cells expressing asTF responded to estrogen treatment by increasing their proliferative rate; iii) E2 stimulated BrCa cell growth *in vivo* when asTF is expressed in these cells.

In our previous work, we determined the changes in global gene expression upon asTF and flTF expression in BrCa cells [21]. The obtained gene expression profile was analyzed using IPA and hinted that asTF and flTF expression is associated with diseases such as cancer (Table 1), which is in line with previous findings [20, 21, 28]. TF isoforms also showed significant associations with neurological and reproductive system disorders (Table 1), warranting further studies of possible links between TF isoforms and these diseases. In addition to asTF's role in cell cycle regulation, IPA results suggest that cell death and motility are also controlled by asTF signalling (Table 2). While flTF induced effects on cellular functions overlap with those induced by asTF, flTF appears to selectively influence cellular morphology (Table 2), which is consistent with the literature [29]. asTF-elicited changes in gene expression show high similarity to those elicited by estradiol treatment (Supplementary Table 1). This indicates that the ER and asTF pathways converge downstream. Associations between estrogen and flTF signalling were less significant compared to those between estrogen and asTF (Supplementary Table 2). It is known that TF expression is controlled by oncogenic genes (i.e. K-ras) and impaired p53 expression [30]. Interestingly, flTF-induced gene expression profiles were similar to tumor suppressor p53-dependent expression profiles. This tumor suppressive phenotype may explain why flTF only marginally impacted BrCa cell proliferation *in vivo* and *in vitro* [21].

An overlap between the estrogen pathway and the asTF pathway was also evaluated in a large BrCa patient cohort. asTF expression was significantly

associated with tumor size and grade in ER+ tumors, yet not in ER- tumors, comprising another line of evidence for a combined effect of these two components on BrCa progression. flTF associated with BrCa stage, but this was not restricted to ER+ tumors. 25% of all BrCa tumors show overexpression of the Her2/neu proto-oncogene, leading to decreased survival rates [31]. Interestingly, IPA analysis pointed towards ERBB2 as a potential modulator of the asTF pathway (Supplementary Table 3). We did not analyse the relationship between these two pathways due to the low number of patients with Her2 overexpression in our cohort (Supplementary table 3).

To further investigate ER and asTF synergy on the cellular level, we performed *in vitro* proliferation assays. Our previous work showed a proliferative advantage of asTF- and flTF-expressing cells over control cells *in vitro* [21] which is divergent from the findings in this paper (Fig. 1A-C). In our earlier studies [21], BrCa cells were cultured in medium containing phenol red, a weak ER activator [32]. Because the presence of phenol red may impact the outcome of assays studying the effects of estrogens on cellular behaviour [33], we have now performed proliferation assays using TF isoform-expressing cells maintained in culture media lacking phenol red.

E2 is the main estrogen produced by ovaries during the reproductive period; high levels of E2 are associated with increased BrCa risk [27, 34]. We here show that even a low dose of E2 (1nM) is sufficient to enhance the proliferation rate of asTF-expressing cells, while a 10-fold higher dose of E2 (10nM) is required to enhance proliferation of flTF-expressing cells. These data suggest that asTF expression increases sensitivity to E2. Moreover, both pathways elevate expression of positive regulators of the cell cycle, e.g. CCNA1 (Supplementary Table 1), [21] and decrease expression of negative cell cycle regulators, e.g. $p21^{KIP}$ [21, 35]. In line with our previous findings, E2-induced proliferation of asTF-expressing cells was dependent on β1 integrin ligation (Fig. 2G). Although these data raise the possibility that E2-dependent proliferation is dependent on asTF, we cannot exclude that the opposite, i.e. asTF-dependent proliferation is dependent on E2, is true,

especially as treatment with ICI-182,780 reduced proliferation rates of asTF-expressing cells to roughly those of control cells treated with ICI-182,780. Further studies are warranted to ascertain whether ER signaling and asTF contribute independently and equally to BrCa cell proliferation, or whether one is required for the other.

In vivo, E2 increased asTF-dependent growth, but not expansion of pcDNA cells. asTF expression in itself was also sufficient to spur tumor growth, which is in contrast with the results that we obtained in *vitro* (Fig. 3A,B). We posit that *in vivo*, asTF-dependent tumor growth in the absence of E2 is likely facilitated by asTF's angiogenic potential (Fig 4E,F) as well as asTF's ability to recruit monocytes and macrophages [21, 23, 24] . Of note, in our previous work we also showed that asTF inhibition using antibody approaches diminished proliferation of the ER- cell line MDA-MB-231-mfp in *vitro* and in *vivo*. [21]. Thus, pathways other than those dependent on estrogen likely synergise with asTF, particularly in aggressive ER- BrCa cell lines.

Our studies have uncovered significant associations between the asTF/E2 synergy and clinical parameters. Unfortunately, a number of important BrCa-related issues, such as asTF's associations with disease biology and/or molecular subtypes, remain to be addressed. It would also be most interesting to determine whether asTF expression impacts loco-regional and/or systemic spread, , but our currently available tools preclude us from drawing conclusions on the involvement of asTF in metastasis.

Nevertheless, metastasis is critically dependent on the migratory potential of malignant cells and we determined that BrCa cell migration, as assayed in a wound closure assay, is asTF-dependent. Further, we showed in our experimental setup that asTF contributes to BrCa cell migration in a manner independent of E2. One interesting aspect of this study is that control (pcDNA) cells showed enhanced migration, but not proliferation, after stimulation with E2, suggesting that E2-induced migration and proliferation likely rely on distinct pathways. Indeed, binding of E2 to ER results in the activation of Src as well as focal adhesion kinase (FAK)/paxillin complexation. This in turn activates signalling pathways involving Rac,

Rho, and PAK-1 that play a critical role in cell migration [36]. In contrast, E2-dependent proliferation is mainly under the control of Src/Shc/ERK pathways that may induce CCNA1 expression and, concomitantly, higher proliferation rates [37]. FAK/Rac-dependent pathways do not appear to require the presence of asTF to elicit migration, whereas asTF is evidently crucial for ERK-controlled proliferation. It needs to be stressed that, aside from cancer cell migration, metastasis is dependent on many different processes. Thus, asTF-potentiated migration does not comprise a direct evidence that asTF promotes metastasis in BrCa; that being said, we recently showed that to be the case *in vivo* in an orthotopic model of pancreatic cancer [24].

Similarly, it would have been of interest to assess whether asTF status could predict overall- or disease-free survival in BrCa patients as a function of hormonal therapy. Unfortunately, the cohort used to identify associations between asTF and clinical parameters was assembled between 1985 and 1994, when hormonal therapy was not a standard treatment option for patients with ER+ tumors; thus, we were not able to assess patient survival as a function of hormonal therapy. Nevertheless, our study does suggest that targeting asTF, either pharmacologically or by means of blocking antibodies, may enhance the effects of hormonal therapy in ER+ BrCa. In fact, a study shows that integrin β1-dependent signalling results in phosphorylation of ER [38]. As ER -dependent gene expression is dependent on estrogen binding, as well as ligand-independent phosphorylation [39], ER could well be the point at which E2 and asTF-dependent signalling converge. Also, integrin β1 appears to be involved in the development of resistance to tamoxifen; thus, inhibiting asTF – a ligand for β1 integrins – could very well reverse this process.

In conclusion, asTF and ER pathways synergize to facilitate proliferation of BrCa cells. Blocking downstream common elements of these two signalling pathways may thus be a novel and effective approach to stem BrCa progression.

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Versteeg, HH. Alternatively spliced tissue factor promotes breast cancer growth in a beta1 integrin-dependent manner. *Proc Natl Acad Sci U S A* 2013**; 110**: 11517-22.
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pcDNA vs. asTF	p-value
cancer	2,92E-10
neurological disease	1,44E-08
skeletal/muscular disorders	1,44E-08
organismal injury and abnormalities	3,62E-08
reproductive system disease	3,62E-08
pcDNA vs. flTF	**p-value**
cancer	6,48E-14
organismal injury and abnormalities	6,48E-14
reproductive system disease	6,48E-14
dermatological diseases and conditions	1,04E-08
neurological disease	3,80E-08

Table 1 Ingenuity Pathway Analysis: top disease states significantly associated with asTF- and flTF-triggered pathways.

pcDNA vs. asTF	p-value
cellular growth and proliferation	8,02E-12
cell death and survival	1,30E-11
cellular movement	1,93E-11
cellular development	6,23E-11
cell cycle	3,14E-07
pcDNA vs. flTF	**p-value**
cellular growth and proliferation	7,05E-15
cell death and survival	8,82E-15
cellular movement	6,68E-10
cell morphology	8,84E-07
cellular assembly and organization	8,84E-07

Table 2 Ingenuity Pathway Analysis: top biological processes significantly associated with asTF- and flTF-triggered pathways.

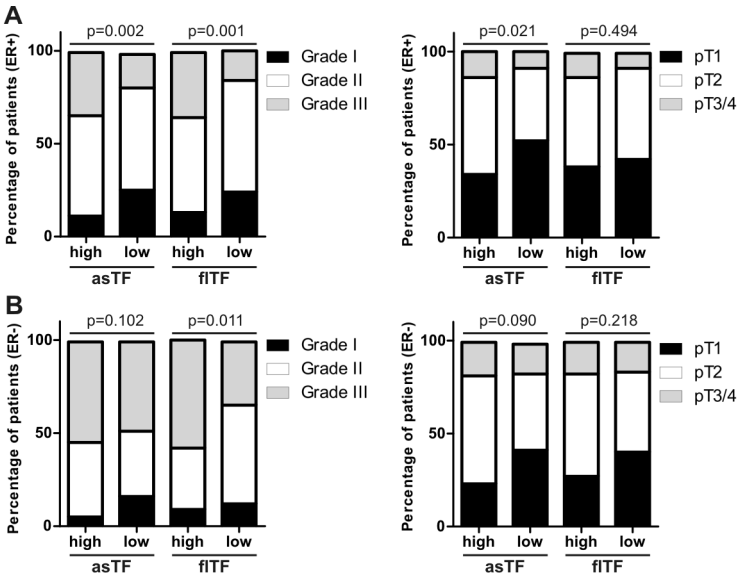

Fig. 1. asTF expression positively associates with BrCa grade and stage in patients with ER+, but not ER- tumors. A) Associations of asTF or flTF expression with BrCa grade and stage in patients with ER+ tumors. B) Associations of asTF or flTF expression with BrCa grade and stage in patients with ER+ tumors. The cohort comprised 574 non-metastatic BrCa patients; X2 statistical tests were used to evaluate associations. Please see supplementary tables 3 and 4 for the associations between asTF or flTF expression and other clinical parameters.

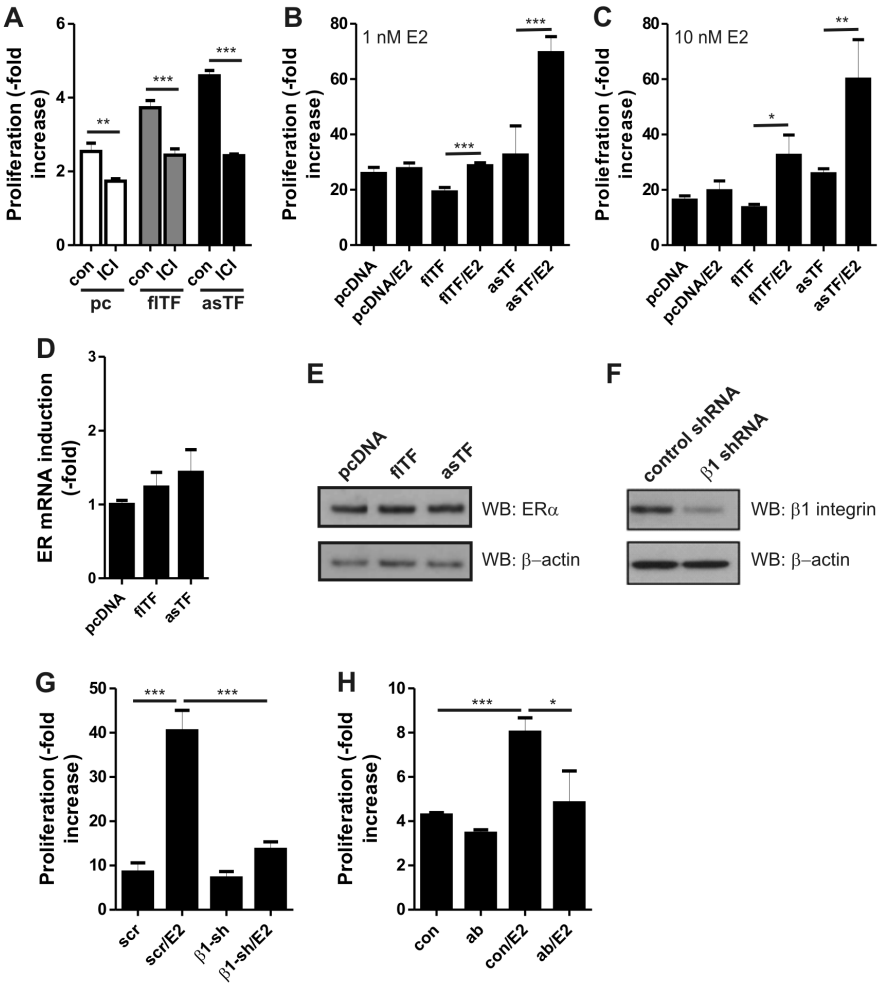

Fig. 2. Estrogens and asTF cooperate to induce BrCa cell proliferation. A) Control, flTF- and asTF-expressing cells were cultured in phenol red-containing media in the presence or absence of ER inhibitor ICI-182,780 (final [C] = 100 nM). After 3 days, proliferation rates were determined using MTT assay. B) Control, flTF- and asTF-expressing cells were cultured in phenol red free medium. Cells were treated with either 1 nM E2 or solvent control (EtOH). After 6 days, proliferation rates were determined using MTT assay. C) As in B, but using 10 nM of E2. D) ERα transcript levels in control, flTF-and asTF-expressing cells were determined using real-time PCR. E) ERα protein levels in control, flTF-and asTF-expressing cells were determined by western blotting. F) β1 integrin was downregulated in asTF-expressing cells using lentiviral shRNA. Scrambled shRNA was used as a control. Reduction of β1 integrin protein levels was verified by western blotting. G) Cells were treated with 1 nM E2 or solvent control. Proliferation rates were assessed using MTT assay at days 3 and 6. *P < 0.05, **P <0.01, and ***P < 0.001. H) Proliferation of asTF-expressing cells in the presence or absence of ER and the asTF-blocking antibody Rb1.

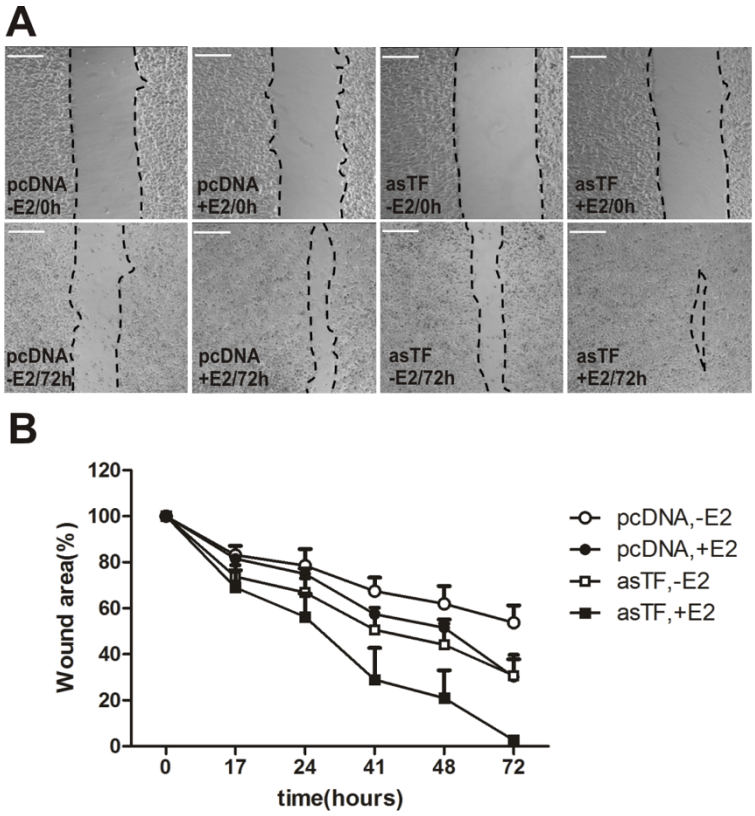

Fig. 3. E2 and asTF independently promote BrCa cell migration. A) Cells were seeded to confluency in silicone inserts. The following day, inserts were removed to leave a gap (demarcated using a dashed line). Gap closure was monitored at regular time intervals. B) The remaining gap area was calculated as percent closure of the area at t=0 by using Image J software. Scale bars: 300μm.

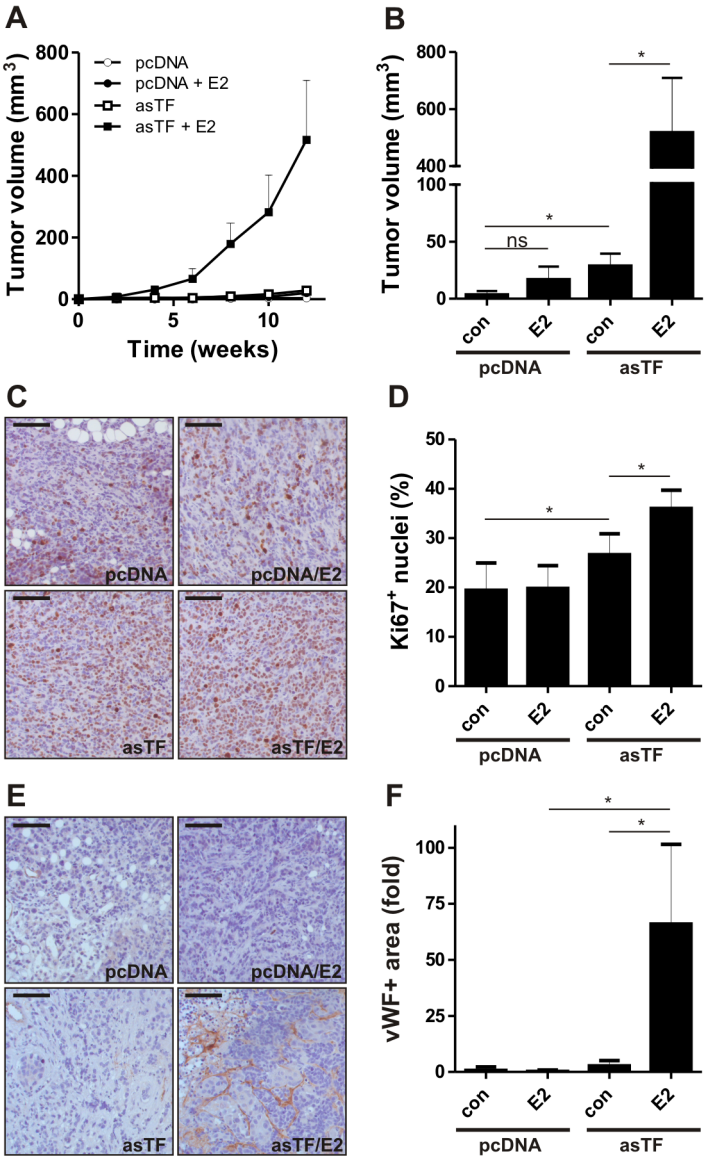

Fig. 4. E2 increases the growth of asTF+ BrCa cells *in vivo*. A) pcDNA or asTF cells were injected into mammary fat pads of NOD/SCID mice. Estrogen pellets were placed subcutaneously. Tumor growth was monitored for 12 weeks by measuring tumor volume. B) Final tumor volumes are shown. C) Proliferating cells were detected using Ki67 staining. Scale bars: 50μm. D) $Ki67^+$ cells were counted and represented as percent of the total cell number. *P < 0.05, **P <0.01, and ***P < 0.001. E) Vessel structures were detected using anti-vWF antibody F) vWF signal strength is shown as fold increase over control, scale bars: 50μm, *P < 0.05.

Supplementary material

pcDNA vs. asTF

upstream regulator	p-value	known target molecules in the dataset
beta-estradiol	1,07E-21	ACSL1, ADK, AEBP1, AP1B1, APOA1, APOE, ATP1B1, BHLHE40, BTG1, C8orf44-SGK3/SGK3, CA12, CAV1, CBL, CCNA1, CCNA2, CD24, CDC45, CITED2, CKB, CLCN3, CNN2, COL4A5, COMT, CRABP2, CTSD, CTSH, CTTN, CYP1B1, DKK1, EDN1, ELOVL2, ERBB3, F12, FABP5, FADS1, FGFR3, G6PD, GAB2, GAL, GHR, GPX3, GSTT1, H19, HBA1/HBA2, HBB, HIST2H2AA3/HIST2H2AA4, HIST2H2BE(includes others), HLA-DRB1, HLA-E, HMGCS1, HSD17B11, HSP90B1, HSPB8, ID1, IFRD1, IGFBP5, IL24, INHBB, IRS2, IRX5, JUN, KITLG, KLF6, KYNU, LDLR, LITAF, MAL2, MARCKS, MGP, MXD4, MYB, MYO1B, MYOF, NELL2, NR3C1, NRP1, ODC1, PBX1, PDZK1, PIK3R1, PIK3R2,PMP22, PPP2CB, PRKCD, PRSS23, PTGES, PTPRK, RANBP1, RAP1GAP, RBL2, RND3, RPRM, RPS4X, S100A6, SAP30, SCNN1A, SLC2A1, SLC2A3, SLC39A6, SLC7A2, SOCS2, SQLE, SSBP2, STC2, TAP2, TFF1, TH, TM4SF1, TMEM164, TNNT1, TSC22D1, XBP1, YWHAH
ESR1	5,24E-14	AHNAK, APOA1, APOE, CAV1, CCNA2, CKB, CLIC3, COMT, CRABP2, CRKL, CTSD, CYP1B1, EDN1, EFEMP1, F12, G6PD, GAL, H19, HIF1A, ID1, IGFBP5, INHBB, IRS2, ISG20, JUN, LDLR, MYB, NQO1, NRCAM, PDZK1, PRKCD, PRSS23, RGS19, RPRM, SLC39A8, SLC7A2, SOCS2, STC2, TFF1, TH,TM4SF1
ERBB2	1,09E-12	ACSL4, ADIPOR2, AHNAK, ANXA2, BHLHE40, CCNA2, CENPE, CPS1, DNAJB6, EDN1, ELF2, ERBB3, ETFB, FAM134B, GHR, GPX3, H19, HBB, HIF1A, HIST1H4A(includes others), HMGB2, HSD17B11, HSPB8, ID1, IGFBP5, IRX3, JUN, LAMP1, LITAF, LRRFIP1, LTBP3, MAPK13, MARCKS, MCCC1, MIF, NDUFAB1, NRP1, PBX1, PTGES, PTPRK, RBL2, RHOB, S100A6, SLC2A1, SPINK4, SQLE ,STMN3, TAP2, TSC22D1, USF2
trans-hydroxytamoxifen	5,34E-12	CD24, COL4A5, CTSH, CYP1B1, ERBB3, HIST2H2AA3/HIST2H2AA4, HIST2H2BE(includes others), INHBB, KLF6, KYNU, LITAF, MXD4, MYO1B, NELL2, PDZK1, PRSS23, RAP1GAP, RND3, SLC39A6, TFF1, TM4SF1
dexamethasone	1,51E-11	ACAT1, ACSL1, AEBP1, AKR1C1/AKR1C2, ALCAM, AP3S1, APOA1, APOE, ATP1B1, BGN, BHLHE40, BTG1, CAV1, CCNA2, CDV3,CFL1, COL4A5, COPA, CPS1, BRABP2, CREG1, CST3, CTSD, CYP1B1, DCTN6, DLK1, DTYMK, DUSP5, EDN1, EIF4E, FABP5, FLNB, G6PD, GAL, GHR, GRN, HBB,HLA-DRB1, HNRNPH1, HAD17B11, ID1, IFI27L2, IFIT1, IFRD1, IGFBP5, INHBB, IRS2, ISG20, ITGB5, JUN, KCNK2, LDLR, LITAF, LMO4, LY6E, MAPK3, MARCKS, MGP, MIF, MT1X, MYB, NAT1, NIPSNAP1, NR3C1, ODC1, PABPC1, PCDH19, PIK3R1, PTGES, PTGFRN, RBL2, RPS23, S100A10, SCNN1A, SLC25A1, SLC2A1, SLC2A3, SNRPC, SOCS2, SPINK4, STOM, TFF1, TIMP1, TNNT1, TSC22D1, UCP2, USF2, WIPF1, XBP1

Supplementary Table 1 Ingenuity Pathway Analysis: top upstream regulators significantly associated with asTF-dependent gene expression. Fischer's exact test was used to calculate the p-values.

pcDNA vs. flTF

upstream regulator	p-value	known target molecules in the dataset
TP53	2,21E-19	ACSL3, ANXA2, ARHGEF2, ARPC1B, ASNS, ATAD2, BCAP31, BTG1, C12orf5, CARHSP1, CAV1, CCND3, CCNG1, CDKN1A, CDT1, CHEK2, CKB, CKS1B, CLIC4, CNN2, CRIP2, CSK, CSTB, CTSD, CYFIP2, DRAM1, DSTN, EBAG9, EDN1, F11R, FHL1, FKBP1A, G6PD, GDF15, H19, H2AFX, H2AFY2, HMGCS1, HMMR, ID1, ID3, IER3, IER5, IFI35, KITLG, KRT8, LSS, MB, MBNL2, MGST2, MTDH, MYH9, MYOF, NRP1, OSGIN1, P2RX4, PAWR, PBK, PDLIM1, PEG10, PERP, PFKM, PFKP, PHLDA3, POLB, PPP1R13B, PRDX6, PRELID1, PRNP, PTP4A1, PTPN11, PTTG1, PYCARD, RFWD2, RHOB, RPRM, RPS27L, SERPINB6, SERPINH1, SHC1, SIVA1, SOCS2, SPDL1, SQLE, STAU1, TAGLN2, TAP1, TAP2, TOP2B, TP53INP1, TYMS, UBL3, UIMC1, ULK1, WWP1, XPNPEP1
beta-estradiol	2,28E-14	ABCA3, ABCC5, ACKR3, ADM, ATAD2, ATP1B1, BACE2, BEX2, BTG1, C1QTNF6, C8orf44-SGK3/SGK3, CAV1, CCND3, CCNG1, CD24, CDKN1A, CITED2, CKB, CKS1B, CLEC2D, CNN2, CRABP2, CSTB, CTSD, CTSL, CXCL12, CYP1B1, DBN1, DECR1, EDN1, EFNB2, ELOVL2, F12, G6PD, GAL, GHR, GJA1, GSTT1, H19, HLA-E, HMGCS1, ID1, IDE, IER3, IKBKG, IL20, INHBB, ITGAV, KITLG, KRT8, KYNU, LAGE3, LAMB1, LMCD1, LMNA, MAL2, MAPT, MATN2, MB, MGP, MUC1, MYB, MYO1B, MYOF, NHP2, NRP1, NSDHL, ODC1, PDZK1, PKIB, PLIN2, PMP22, PRSS23, PTP4A1, PTPN11, PTPRK, PTTG1, RANBP1, RPL39L, RPRM, SBK1, SCNN1A, SLC2A3, SLC39A6, SLC6A14, SLC7A2, SOCS2, SQLE, SSR2, STK3, TAP2, TFPI, TM4SF1, TMED9, TNNT1, TSC22D1, TXNRD1, YWHAH
MYC	3,61E-11	ADM, ALCAM, ARF3, ASNS, B4GALT7, BMI1, C9orf3, CAV1, CCND3, CCT3, CDKN1A, CLIC4, CRIP2, CSTB, CTSD, CYFIP2, DBN1, DKC1, DSTN, EDN1, GAMT, GDI1, GJA1, HIST1H4A (includes others), HLA-A,HLA-E, HNRNPAB, ID1, ID3, IER3, IFI35, IFIT1, LAMP2, LGALS1, MAPKAPK5, METAP2, MGAT1, MGP, MGST3, MYO1B, NBN, ODC1, PERP, PFKM, PFKP, PHB2, PLS3, PMP22, PRPH, RANBP1, RHOB, RPL13, RPS15A, RPS23, SERPINH1, SLC2A3, TAGLN2, TNS3, TYMS, UGT1A6
trans-hydroxytamoxifen	4,13E-10	ABCC5, CD24, CTSL, CXCL12, CYP1B1, DBN1, IER3, INHBB, KYNU, LAMB1, MAPT, MATN2, MB, MUC1, MYO1B, PDZK1, PRSS23, SLC39A6, TM4SF1
NKX2-3	5,79E-10	ADAP1, ADM, ASNS, BCYRN1, BTG1, CBS, EDN1, FAM46A, GCA, GDF15, GHR, HIST2H2AC, HMMR, LY6E, LYPD1, MYO5A, NETO2, PAWR, RPS23, TAP1, TAP2, TFF3, UBE2L6, WRB, ZNFX1

Supplementary Table 2 Ingenuity Pathway Analysis: top upstream regulators significantly associated with flTF-dependent gene expression. Fischer's exact test was used to calculate the p-values.

	ER+ asTF high		ER+ asTF low		p-value	ER- asTF high		ER- asTF low		p-value
	N	%	N	%		N	%	N	%	
Total	**196**	**100**	**81**	**100**		**126**	**100**	**38**	**100**	
Age (y)										
<40	10	5.1	2	2.5		15	11.9	3	7.9	
40-60	85	43.4	45	55.6	0.149	64	50.8	27	71.1	0.087
>60	101	51.5	34	42.0		47	37.7	8	21.1	
Missing	0		0			0		0		
Grade										
I	22	11.4	21	25.9		7	5.6	6	16.2	
II	105	54.4	45	55.6	**0.002**	51	40.5	13	35.1	0.109
III	66	34.2	15	18.5		68	54.0	18	48.6	
Missing*	3		0			0		1		
Histologic type										
Ductal	178	92.2	73	90.1	0.566	116	92.1	32	86.5	0.302
Lobular	15	7.8	8	9.9		10	7.9	5	13.5	
Missing*	3		0			0		1		
Tumor stage										
pT1	64	33.9	41	51.9		29	23.4	15	41.7	
pT2	99	52.4	31	39.2	**0.021**	72	58.1	15	41.7	0.090
pT3/4	26	13.8	7	8.9		23	18.5	6	16.7	
Missing*	7		2			2		2		
Nodal stage										
pN0	109	57.7	46	58.2	0.933	54	43.5	18	47.4	0.678
pN+	80	42.3	33	41.8		70	56.5	20	52.6	
Missing*	7		2			2		0		
PgR receptor										
Negative	50	25.6	16	19.8	0.296	91	72.8	26	68.4	0.599
Positive	145	74.4	65	80.2		34	27.2	12	31.6	
Missing*	1		0			1		0		
HER2 status†										
No overexpression	162	95.9	65	98.5	0.318	88	76.5	28	80.0	0.667
Overexpression	7	4.1	1	1.5		27	23.5	7	20.0	
Missing*	27		15			11		3		

Supplementary Table 3 Association of asTF expression levels with patient and tumor characteristics in ER+ and ER- specimens.
N, axillary lymph node; T, tumor.
*During specimen processing, some tumor tissue samples were lost leading to a smaller patient number per staining.
†HER2 status was not available for all patients.

	ER+ flTF high		ER+ flTF low		p-value	ER- flTF high		ER- flTF low		p-value
	N	%	N	%		N	%	N	%	
Total	**217**	**100**	**96**	**100**		**124**	**100**	**57**	**100**	
Age (y)										
<40	11	5.1	6	6.2		15	12.1	9	15.8	
40-60	101	46.5	40	41.7	0.703	67	54.0	31	54.4	0.741
>60	105	48.4	50	52.1		42	33.9	17	29.8	
Missing	0		0			0		0		
Grade										
I	28	13.1	23	24.2		11	8.9	7	12.5	
II	111	51.9	57	60.0	**0.001**	41	33.1	30	53.6	**0.011**
III	75	35.0	15	15.8		72	58.1	19	33.9	
Missing*	3		1			0		1		
Histologic type										
Ductal	196	91.6	84	88.4	0.378	116	93.5	47	83.9	**0.041**
Lobular	18	8.4	11	11.6		8	6.5	9	16.1	
Missing*	3		1			0		1		
Tumor stage										
pT1	81	38.4	39	41.9		33	27.0	22	40.0	
pT2	102	48.3	49	49.5	0.494	67	54.9	24	43.6	0.218
pT3/4	28	13.3	8	8.6		22	18.0	9	16.4	
Missing*	6		0			2		2		
Nodal stage										
pN0	121	58.2	57	60.0	0.802	55	45.1	32	57.142.9	0.149
pN+	87	41.8	38	40.0		67	54.9	24		
Missing*	9		1			2		1		
PgR receptor										
Negative	53	24.7	20	21.5	0.662	94	77.0	34	59.6	**0.021**
Positive	162	75.3	73	78.5		28	23.0	23	40.4	
Missing*	2		3			2		0		
HER2 status†										
No overexpression	171	95.5	76	98.7	0.286	89	77.4	36	75.0	0.839
Overexpression	8	4.5	1	1.3		26	22.6	12	25.0	
Missing*	38		19			9		9		

Supplementary Table 4 Association of flTF expression levels with patient and tumor characteristics in ER+ and ER- specimens.
N, axillary lymph node; T, tumor.
*During specimen processing, some tissue samples were lost leading to a smaller patient number per staining.
†HER2 status was not available for all patients.

Chapter 5 – Ectopic Factor VII associates with survival and regulates tumor progression in breast cancer

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Abstract

Tissue Factor (TF), the initiator of coagulation, is expressed on cancer cells and TF binding to Factor VII (FVII), produced in the liver and present in the microenvironment induces angiogenesis and tumor growth. Here we report that FVII is also expressed in breast cancer specimens, and is associated with relapse free survival and higher tumor grade. In breast cancer cell lines, ectopic FVII induces expression of pro-angiogenic markers, drives tumor cell invasion and produces gene expression profiles reminiscent of the TGFβ pathway. In vivo, ectopic FVII enhanced tumor growth and liver metastasis, but not lung metastasis. Finally, ectopic FVII drives tumor growth and metastasis independently of circulation FVII. We propose here that ectopic FVII is a major driver of breast cancer progression, by efficiently inducing TF:FVII:PAR-2 signaling on tumor cells. Blockade of tumor FVII might prove a novel angle for new therapeutic strategies in breast cancer patients.

Introduction

Increasing evidence points towards a role for a procoagulant microenvironment in cancer progression. Coagulation factors such as Factor VII (FVII) and Factor X (FX), produced in the liver and present in the circulation, are part of this tumor milieu and can bind the initiator of extrinsic coagulation Tissue Factor (TF), which is broadly expressed by cancer cells and associated with higher tumor grade and reduced survival in cancer patients.[1–3] While TF in complex with FVII and FX in normal physiology produces thrombin production to form a blood clot after vascular damage, TF also functions as a cellular co-factor to induce FVII-dependent cleavage of the Protease-Activated Receptor (PAR)2 .[4] PAR2 activation leads to an inflammatory and angiogenic response that facilitates primary tumor growth.[5,6] Whether FVII is also expressed by breast tumors and contributes to tumor progression is currently unknown.

There is evidence that, apart from the liver, cancer cells produce FVII through hypoxia-inducible factor 2α (HIF2α)[7] or EGFR-dependent

pathways *in vitro*.[8] Ectopic FVII expression increases cell migration and invasion in a TF dependent manner.[9] Nevertheless, it is unknown whether FVII is produced in human cancers *in situ* and this lead us to investigate whether FVII is expressed in tumor specimens and contributes to cancer progression in breast cancer patients. Here, we show that breast tumors produce FVII, and this is associated with higher tumor grade and diminished relapse free survival. Furthermore, we show that ectopic FVII expression enhances tumor cell aggressiveness both *in vitro* and *in vivo*, independently of circulation FVII.

Methods

Patient Material

574 breast tumor specimens were collected in the Leiden University Medical Center between 1985 and 1994, all patients presented with non-metastasized breast cancer and were undergoing surgery.[10] Patients characteristics were recorded: age, tumor grade, histological type, TNM (TNM classification of malignant tumors, Union for International Cancer Control), stage, local and systemic therapy, loco-regional or distant tumor recurrence, survival, and expression of estrogen receptor (ER), progesterone receptor (PgR) or human epidermal growth factor receptor 2 (HER2). Median follow-up time was 17.9 years (range: 0.01-23.5). Approval was obtained from the Leiden University Medical Centre Medical Ethics Committee. 4 μm tissue samples were cut from a tissue micro array containing formaline-fixed paraffin-embedded tumors in triplicate.
4 μm tissue samples were cut from a tissue micro array containing formaline-fixed paraffin-embedded tumors in triplicate. In short, the sections were de-paraffinized, rehydrated and endogenous peroxidase activity was blocked using 0.3% H_2O_2. Citrate antigen retrieval was performed, following 1 hour blocking using 5% BSA in PBS. Sections were incubated with 1 μg/ml antibody in 5% BSA in PBS overnight. Sections were washed with PBS and incubated with Envision (Dako, Glostrup, Denmark) for 1 hour. Sections were washed with PBS, staining was

visualized after 10 minute DAB (Dako, Glostrup, Denmark) treatment, counterstained with Hematoxilin, dehydrated and covered.

Cells culture, transfections and proliferation assays

Cell lines were acquired from ATCC (Manassas, VA, USA), and cultured in RPMI (PAA, Pashing, Austria) supplemented with 10% FBS, 100 mM L-Glutamine and 1% penicillin/streptomycin. The HCC1937mfp cell line was established by injection of $1x10^6$ HCC1937 cells into the mammary fat pad of a NOD-SCID mouse and subsequent re-isolation of cells from the tumor. An FRT site was introduced in MDA-MB-231 cells using Lipofectamine 2000 (Invitrogen, Calrsbad, CA, USA) and clones were selected using 100 μg/ml Zeocin and verified by β-galactosidase western blot expression. MDA^{FRT} cells were transfected with pOG44 yielding transient recombinase expression in the presence of pcDNA5-FRT (empty vector control) or pcDNA5-FVII, Cells undergoing homologous recombination were selected using 150 μg/ml hygromycin. Cell proliferation was determined by a methyl-tetrazolium bromide mitochondrial activity (MTT) assay as described before.[11]

Western Blotting

Cells were lysed in sample buffer (Life, Carlsbad, CA, USA), and samples were run on 6-18% gradient gels and transferred to PVDF membranes. Membranes were blocked using 5% non-fat milk powder and incubated o/n with the appropriate antibodies. Membranes were washed and incubated with HRP-conjugated secondary antibodies. Western Lightning ECL (PerkinElmer, Shelton, CT, USA) was used to visualize protein bands on Kodak film (X-sanatec, Genk, Belgium).

FXa Generation

Cells were seeded overnight in a 48-wells plate and serum-starved for 24 hours to allow for FVII synthesis and transport to the cell membrane. 231-pcDNA and 231-FVII cells were pre-incubated with 50 μg/ml 3G12 or control IgG_1 for 10 minutes, HCC1937 cells were pre-incubated with 100 nM

Ixolaris or solvent control. 100 nM FX was added for 30 minutes, after which the reaction was quenched and generated FXa was determined with the chromogenic substrate Spectrozyme Xa (American Diagnostica, Greenwich, CT, USA).

Cell migration and invasion assays

$3x10^4$ cells were seeded in the top compartment of Biocoat invasion (matrigel-coated) or migration (no matrigel) chambers (BD Biosciences, San Jose, CA, USA) in RPMI without FBS. Cells were mixed with 50 μg/ml antibody. RPMI supplemented with 10% FBS was used as chemo-attractant in the lower compartment. After 16 hours, invasion was quantified by crystal violet cell staining. For HCC1937 cell migration $5x10^4$ cells were seeded in silicon cell culture inserts (Ibidi, Munich, Germany) and allowed to attach overnight. Inserts were removed and fresh RPMI medium containing 0, 50 or 200 nM Ixolaris was added. Cells were allowed to migrate for 20 hours, after which the area covered was quantified using ImageJ software.

In vivo studies

All mice experiments were approved by the Leiden University Medical Centre animal experimental committee. 6 weeks old female NOD SCID mice were acquired from the Jackson Laboratory (Bar Harbor, ME, USA). $1x10^6$ MDA-MB-231 cells transfected to express human FVII or empty vector (pcDNA) were injected into the fourth mammary fat pad, and tumor size was measured in time using calipers. Tumor size was calculated as $0.5 * width^2 * length$. After defined times the mice were sacrificed, tumor, blood and lungs were collected. Tumors and lungs were formalin fixed, embedded in paraffin, and 5 μm sections were cut for further immunohistochemistry as described above.

Results

Ectopic FVII expression associates unfavorably with clinical parameters

To explore the role of ectopic FVII in breast cancer we analyzed FVII protein expression in a tissue array of 574 breast cancer patients and found that 39% of the tumor specimens were positive (fig 1A). Analysis of FVII mRNA expression in selected specimens from this array confirmed that FVII is produced by tumor cells and not taken up from the circulation (fig 1D). FVII protein expression associated with tumor grade (fig. 1E) and T-status (table 1). Interestingly, FVII expression was negatively associated with ER- and PgR-status, suggesting that FVII expression predominantly takes place in triple negative or HER^+ breast cancer (table 1). FVII expression also associated with decreased relapse free survival in patients (fig 1H). We observed a strong association with positive FVII and TF staining (fig 1F). As FVII in breast cancer is expected to interact with TF expressed on tumor cells we investigated the combination of both TF and FVII expression in breast cancer, compared to either or both TF and FVII negative. The combination of TF and FVII expression significantly associated with tumor grade (fig 1G), stage, and nodal stage (table 2). Interestingly, expression of both TF and FVII by breast tumors is associated with a further decrease in relapse-free survival compared to FVII alone (fig 1I). Finally, we found that 355 (74.1%) out of 479 tumor specimens stained positive for PAR-2, and positively associated with tumor grade as well as Progesterone Receptor (PgR) status and HER-2 overexpression (table 3).

FVII expression drives expression of pro-angiogenic and invasive markers

FVII and TF mRNA expression was further examined in a panel of breast cancer cell lines (sup fig 1AB**).** We then chose the cell line MDA-MB-231 cell line for its high TF expression, and transfected this line with human FVII cDNA using Invitrogen's Flp-in system, as described before.[2] This resulted in a cell line stably expressing both TF and FVII (fig 2A, sup fig 1C). FVII expression resulted in a decrease in TF both at the mRNA and protein level.

TF/FVII complexes in these cells were proteolytically active as 231-FVII cells, but not control (pcDNA) cells, showed potent FXa generation in the absence of exogenous FVII (fig 2C, sup fig 1D). A panel of monoclonal FVII antibodies was screened for anti-FVIIa activity (sup fig 2A) and MAb 3G12 was selected as it detects FVII on Western blot (sup fig 2B) and strongly inhibits human FVIIa proteolytic activity (sup fig 2C) but not murine FVIIa. MAb 3G12 completely inhibited activation of FX on 231-FVII cells (fig 2C). Adding 1 nM FVIIa to 231-FVII cells did not increase FXa levels (sup fig 1D), suggesting that TF on the cell surface was saturated with cell-produced FVII.

FVII expression did not significantly induce cell proliferation (fig 2D), after which we investigated responses typically associated with TF/FVII signaling such as expression of angiogenic mediators and matric metalloproteinases. IL-8 and CXCL-1, but not VEGF, were upregulated in 231-FVII cells compared to 231-pcDNA control cells **(**fig 2E**)**. Importantly, IL-8 showed a stronger induction in 231-FVII cells than 231-pcDNA cells exposed to recombinant FVIIa (sup fig 1E).

We aimed to identify a breast cancer cell line that expresses both TF and FVII endogenously. The cell line HCC1937, isolated from a primary ductal mamma carcinoma, expresses abundant levels of both TF and FVII (sup fig 1A,B,3A). Exposure to Ixolaris, a tick-derived TF:FVII inhibitor,[12] decreased endogenous TF/FVII-dependent FXa generation (sup fig 3B), but not proliferation rates (sup fig 3C**)**. Besides inhibiting coagulation, Ixolaris also a potently inhibits TF:FVIIa signaling.[13] Ixolaris induced a dose-dependent decrease of expression of the TF:FVIIa downstream targets VEGF, IL-8 and CXCL-1 in HCC1937 cells (sup fig 3D). Furthermore, HCC1937 cell migration was dose-dependently reduced after Ixolaris exposure (sup fig 3E). We observed that HCC1937 cells do not potently expand *in vivo*, although tumors do occasionally form. We re-isolated cells from one such tumor (HCC1937mfp) showing TF and FVII amounts comparable to other FVII expressing breast cancer cell lines (fig 3A). TF and FVII expressed by

HCC1937mfp cells readily activated FX to FXa, which could be inhibited with TF or FVII-blocking antibodies (fig 3B). Exposure to inhibiting antibodies against TF, FVII or PAR-2 did not influence cell proliferation after five days compared to an IgG control (fig 3C). As induced FVII expression increased matrigel invasion in MDA-MB-231 cells, we determined whether inhibition of FVII, TF, or PAR-2 reduced invasion in HCC1937mfp cells. Figure 3D shows that matrigel invasion could be significantly inhibited by antibody inhibition directed against TF, FVII, or PAR-2.

FVII expression enhances tumor growth *in vivo*

We next assessed effects of FVII on tumor growth in an orthotopic breast cancer model. 231-FVII cells yielded significantly larger tumors compared to 231-pcDNA cells (fig 4A). We observed that the number of proliferating tumor cells per field were comparable between 231-pcDNA and 231-FVII derived tumors (fig 4B) but 231-FVII cells yielded significantly larger $Ki67^{+}$ positive areas at the periphery of the tumor (fig 4C). Furthermore, an increase in intra-tumoral $CD\text{-}31^{+}$ vessel density was observed in 231-FVII tumors compared to 231-pcDNA (fig 4D), confirming our *in vitro* observation that FVII expression induces production of pro-angiogenic peptides, but not cell proliferation. 231-pcDNA or 231-FVII cells were then co-injected with specific TF, FVII or IgG control antibodies. Both TF and FVII inhibition decreased tumor volume in the 231-FVII groups, while the 231-pcDNA groups were not significantly affected by TF or FVII inhibition (fig 4E), confirming that *in vivo* expansion of 231-FVII cells was dependent on both TF and FVII. Furthermore, HCC1937mfp cells implanted with the FVII antibody 3G12 or TF antibody 10H10 resulted in a significantly reduced tumor volume compared to tumors treated with an isotype-matched IgG (fig 4F).

Ectopic FVII drives tumor development and metastasis independently of circulation FVII

As FVII is present in the circulation, we set out to establish how tumor-derived and circulation FVII respectively contribute to tumor development. Mouse FVII and human TF are compatible in coagulation activation and murine xenograft models are therefore suitable to study the interaction between tumor-derived TF, tumor FVII and circulation FVII. FVII knockout in mice produces lethality at the embryonic/perinatal stage,[14] thus circulating FVII was silenced using siRNA directed against the *f7* gene in the liver (sup fig 4A), the physiological site of its production. Subsequently, 231-pcDNA or 231-FVII tumor cells were implanted in the mammary fat pad as outlined in Fig. 5A. f7 silencing in the liver significantly inhibited Tumor development of 231-pcDNA cells but did not affect expansion of FVII cells *in vivo* (fig 5B). f7 silencing was successful throughout the experiment (sup fig 4A); FVIIa activity was significantly reduced, the Pro-thrombin time (PT) was prolonged (sup fig 4B) while the activated partial Thromboplastin time (aPTT) was unaffected (sup fig 4C). The FVII knockdown specificity was confirmed on mRNA level at the end of the experiment (sup fig 4D), expression of other coagulation factors in the liver was not affected.

Expression of tumor FVII resulted in significantly increased human mRNA in the liver, (fig 5C), as well as increased macroscopic foci (sup fig 4E) and cytokeratin$^+$ micro metastases (sup fig 4F). Thus, FVII expression in MDA-MB-231 cells enhanced liver metastasis. In complete contrast with our expectation, F7 siRNA treatment significantly increased liver metastasis both for 231-pcDNA and 231-FVII compared to the control siRNA. This increase in metastasis was not observed in lung tissue (sup fig 4G,4H,4I).

Discussion

In this study, we provided clear evidence that FVII is widely expressed in breast cancer alongside TF expression, and significantly enhances disease progression via PAR-2 dependent TF signaling. We also show that tumor-

FVII significantly increases tumor growth *in vivo*, and that this effect is independent of FVII levels in the circulation. By associating FVII staining with FVII mRNA levels, we excluded the possibility that circulation FVII internalized by tumor cells explains our findings in the patient cohort. Evidence for FVII expression in breast cancer has so far been sporadic and its role in tumor progression has not been investigated in detail. FVII expression in a breast cancer patient cohort has been associated with androgen receptor (AR) expression, and AR activation lead to FVII upregulation in breast cancer cells.[15] Koizume *et al*. have shown that FVII mRNA is expressed in breast cancer cell lines, and that ovarian tumor cell migration and invasion is enhanced by FVII expression.[9] Furthermore, FVII mRNA was upregulated under hypoxic conditions.[16] Exposure of TF-bearing tumor cells to circulation-derived FVII is limited prior to the development of an intra-tumoral blood vessel network. Therefore, if FVII is expressed during early phases of tumor development, this can enhance TF signaling when the supply of circulation FVII is still limited. Besides TF upregulation by tumor cells under hypoxic stress,[17,18] upregulation of FVII could enable TF:FVII signaling, triggering angiogenesis and tumor cell migration.

The role of TF:FVII:PAR2 signaling on breast cancer cells has been studies in detail in *in vitro* breast cancer models,[19] but these models rely on TF and PAR2 expression, while FVII(a) is added under culture conditions. The presence of PAR2 expression in breast cancer cell models has been established, and PAR2 protein expression in breast tumor specimens has been reported.[20,21] But so far the presence of PAR2 in breast cancer has not been associated with clinical outcome. Here we report that PAR2 in breast cancer specimens associates with worse clinical outcome.

The role of TF signaling in cancer progression has been well established, mainly by promoting angiogenesis via PAR-2. TF signaling activity is dependent on the formation of the TF:FVIIa binary complex to activate PAR-2. *In vitro* studies in TF signaling make use of added recombinant FVIIa in the nM range, whereas murine models rely on the fact that mouse FVII from the circulation has comparable efficacy in binding human TF as

compared to human FVII. Here we show that FVII is widely expressed in breast cancer specimens alongside TF and PAR2, and that FVII expression significantly enhances disease progression. We also show that FVII expressed by tumor cells is capable to induce robust TF signaling in *in vitro* models.

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Table 1. Associations of FVII with patients and tumor characteristics.

Characteristic	Total N (%)	FVII - N (%)	FVII + N (%)	*P*
Total	331 (100)	201 (100)	130 (100)	
Grade				
I	42 (12.7)	33 (16.4)	9 (6.9)	
II	165 (49.8)	110 (54.7)	55 (42.3)	<0.001
III	118 (35.6)	55 (27.4)	63 (48.5)	
Missing	6 (1.8)	3 (1.5)	3 (2.3)	
Histological type				
Ductal	300 (90.6)	179 (89.1)	121 (95.3)	0.057
Lobular	26 (7.9)	20 (9.9)	6 (4.7)	
Missing	5 (1.5)	2 (1.0)	3 (2.3)	
T-status				
T1	126 (38.1)	87 (43.3)	39 (30.0)	
T2	157 (47.4)	81 (40.3)	76 (58.5)	0.009
T3/4	41 (12.4	27 (13.4)	14 (10.8)	
Missing	7 (2.1)	6 (3.0)	1 (0.8)	
N-status				
N0	180 (54.4)	114 (56.7)	66 (50.8)	0.359
N1-3	144 (43.5)	84 (41.8)	60 (46.2)	
Missing	7 (2.1)	3 (1.5)	4 (3.0)	
ER-status				
Negative	123 (37.2)	63 (31.3)	60 (46.2)	0.011
Positive	195 (58.9)	128 (63.7)	67 (51.5)	
Missing	13 (3.9)	10 (5.0)	3 (2.3)	
PgR-status				
Negative	125 (37.8)	60 (29.9)	65 (50.0)	0.001
Positive	190 (57.4)	127 (63.2)	63 (48.5)	
Missing	16 (4.8)	14 (6.9)	2 (1.5)	
Her2-status				
Overexpression -	221 (66.8)	129 (64.2)	92 (70.8)	0.166
Overexpression +	29 (8.7)	13 (6.5)	16 (12.3)	
Missing	81 (24.5)	59 (29.3)	22 (16.9)	
Tissue Factor				
low expression	101 (30.5)	87 (43.3)	14 (10.8)	<0.001
high expression	201 (60.7)	91 (45.3)	110 (84.6)	
Missing	29 (8.8)	23 (11.4)	6 (4.6)	

Table 2. Patients and tumor characteristics of TF/FVII+ tumors compared to tumor negative for either or both TF and FVII.

	Total N (%)	TF-/FVII-, TF+/FVII-, TF-/FVII+ N (%)	TF/FVII + N (%)	*P*
Grade				
I	38 (11.5)	30 (15.6)	8 (7.3)	
II	152 (95.8)	109 (56.8)	43 (39.0)	0.001
III	107 (38.8)	51 (26.6)	56 (51.0)	
Missing	5 (1.9)	2 (1.0)	3 (2.7)	
Histological type				
Ductal	271 (90.6)	168 (87.5)	103 (93.6)	0.291
Lobular	26 (7.5)	22 (11.5)	4 (3.6)	
Missing	5 (1.9)	2 (1.0)	3 (2.7)	
T-status				
T1	112 (35.8)	78 (40.6)	34 (30.9)	
T2	114 (49.5)	82 (42.7)	62 (56.4)	<0.001
T3/4	39 (12.7)	26 (13.5)	13 (11.8)	
Missing	7 (2.0)	6 (3.1)	1 (0.9)	
N-status				
N0	169 (55.1)	112 (58.3)	57 (51.8)	<0.001
N1-3	127 (42.6)	78 (40.6)	49 (44.5)	
Missing	6 (2.3)	2 (1.0)	4 (3.6)	
ER-status				
Negative	115 (40.0)	63 (32.8)	52 (47.2)	0.725
Positive	184 (59.0)	127 (66.1)	57 (51.8)	
Missing	3 (1.0)	2 (1.0)	1 (0.9)	
PgR-status				
Negative	120 (41.7)	66 (34.4)	54 (49.1)	0.744
Positive	175 (56.3)	120 (62.5)	55 (50.0)	
Missing	7 (2.0)	6 (3.1)	1 (0.9)	
Her2-status				
expression-	224 (74.1)	143 (74.5)	81 (73.6)	0.401
expression+	30 (10.5)	16 (8.3)	14 (12.7)	
Missing	48 (15.4)	33 (17.2)	15 (13.6)	

Table 3. Associations of PAR2 with patients and tumor characteristics.

Characteristic	Total N (%)	PAR-2 - N (%)	PAR-2 + N (%)	*P*
Total	479 (100)	124 (25.9)	355 (74.1)	
Grade				
I	66 (13.8)	34 (27.4)	32 (9.0)	
II	238 (49.7)	61 (49.2)	177 (49.9)	<0.001
III	171 (35.7)	28 (22.6)	143 (40.2)	
Missing	4 (0.8)	1 (0.8)	3 (0.8)	
Histological type				
Ductal	431 (90.0)	106 (85.5)	325 (91.5)	0.043
Lobular	44 (9.2)	17 (13.7)	27 (7.6)	
Missing	4 (0.8)	1 (0.8)	3 (0.8)	
T-status				
T1	167 (34.9)	54 (43.5)	113 (31.8)	
T2	238 (49.7)	50 (40.3)	188 (53.0)	0.072
T3/4	62 (12.9)	16 (12.9)	47 (13.2)	
Missing	12 (2.5)	5 (4.0)	7 (2.0)	
N-status				
N0	244 (50.9)	66 (53.2)	178 (50.1)	0.904
N1-3	220 (45.9)	53 (42.8)	167 (47.1)	
Missing	15 (3.1)	5 (4.0)	10 (2.8)	
ER-status				
Negative	175 (36.5)	38 (30.6)	137 (38.6)	0.105
Positive	291 (60.8)	83 (66.9)	208 (58.6)	
Missing	13 (2.7)	3 (2.4)	10 (2.8)	
PgR-status				
Negative	201 (42.0)	37 (29.8)	164 (46.2)	0.002
Positive	262 (54.7	82 (66.1)	180 (50.7)	
Missing	16 (3.3)	5 (4.0)	11 (3.1)	
Her2-status				
No Overexpression	354 (73.9)	89 (71.8)	265 (74.6)	0.012
Overexpression	40 (8.4)	3 (2.4)	37 (10.4)	
Missing	85 (17.7	32 (25.8)	53 (14.9)	

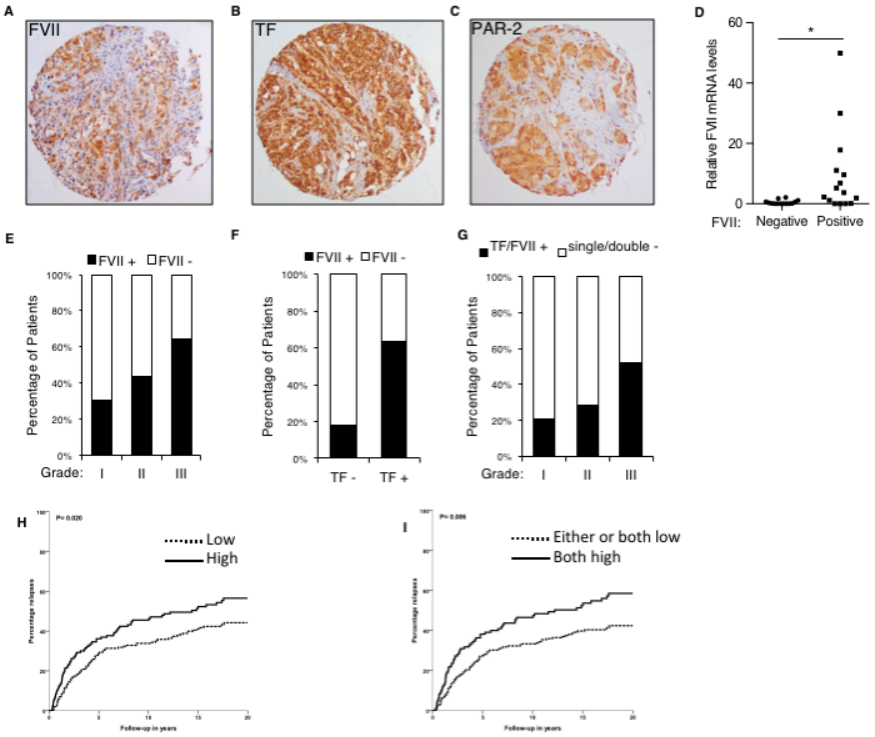


Figure 1. TF and FVII expression in breast cancer specimens associated with clinical parameters. Representative staining of FVII (**A**), TF (**B**) and PAR-2 (**C**). **D** Specimens positive for FVII staining also showed significantly higher FVII mRNA levels. **E** FVII staining associated positively with a higher tumor grade. **F** FVII staining associated with (full length) TF staining. **G** Positivity for both TF and FVII associated strongly with a higher tumor grade, compared to single or double negative staining. **H** FVII staining associated significantly with relapse free survival, while this effect became more prominent when tumors positive for both TF and FVII were compared to single or double negative stainings (**I**).

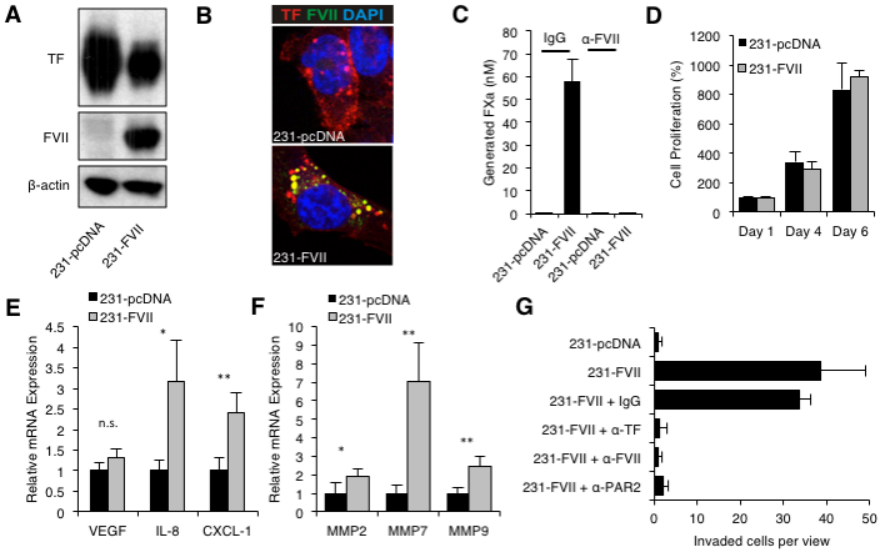


Figure 2. Expression of FVII in MDA-MB-231 increases expression of pro-angiogenic markers and cell invasiveness. **A** MDA-MB-231 cells were transfected with FVII cDNA, as shown by Western blot. **B** FVII expressed in MDA-MB-231 cells show a granular pattern. **C** 231-FVII cells generate ample amounts of FXa, which is inhibited by the FVII antibody 3G12. **D** Cell proliferation of MDA-MB-231 cells is not dependent on FVII expression. **E** FVII expression increased IL-8 and CXCL-1 mRNA levels, but not VEGF. **F** Expression of MMP2, 7 and 9 mRNA was increased in 231-FVII compared to 231-pcDNA. **G** FVII expression increased matrigel invasion, and this effect was inhibited by the presence of specific TF, FVII or PAR-2 inhibiting antibodies.

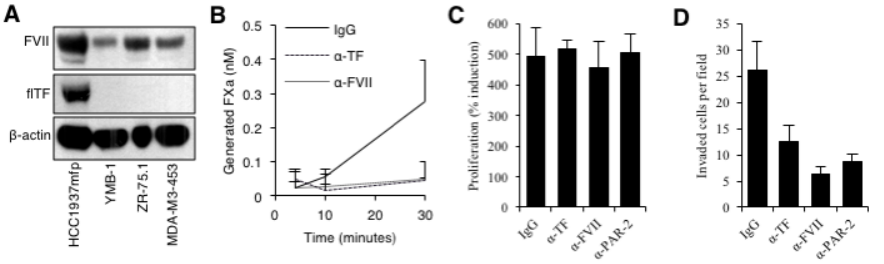


Figure 3. HCC1937mfp expresses functional TF and FVII. **A** Western blot showing FVII and flTF protein expression in breast cancer cell lines, HCC1937mfp co-expresses FVII and flTF. **B** HCC1937mfp cells can generate FXa, which is inhibited by specific the TF antibody 5G9 or the FVII antibody 3G12. **C** Cell proliferation is not influenced by exposure to TF, FVII, or PAR-2 inhibiting antibodies. **D** Matrigel invasion is inhibited by TF, FVII and PAR-2 inhibiting antibodies compared to a IgG control.

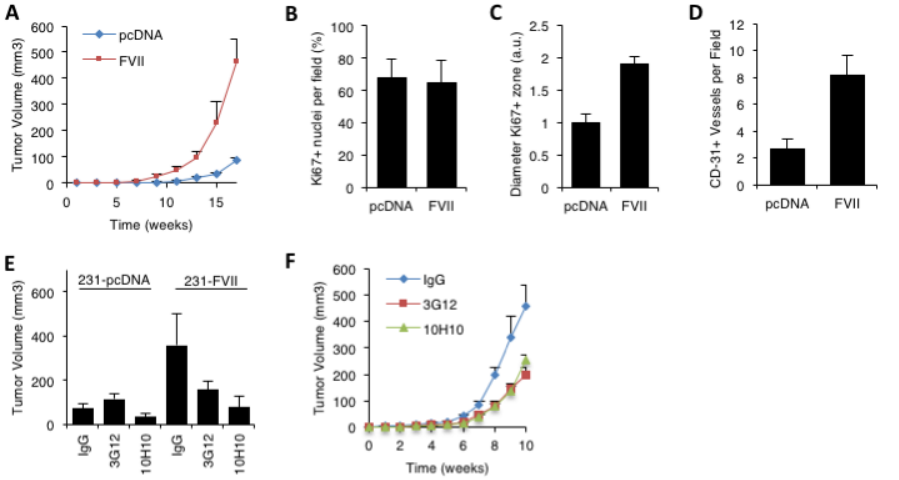


Figure 4. Ectopic FVII expression enhances tumor development *in vivo*. **A** 231-FVII yields larger tumor volume compared to 231-pcDNA. **B** Ki67+ tumor cell nuclei do not differ significantly between 231-pcDNA and 231-FVIi tumors. **C** 231-FVII derived tumors show larger Ki-67+ tumor areas than 231-pcDNA tumors. **D** An increase in CD-31+ tumor vessels is observed in 231-FVII tumors compared to 231-pcDNA. **E** Antibody inhibition of TF or FVII significantly decreased 231-FVII but not 231-pcDNA tumor volume. **F** HCC1937mfp tumor growth is diminished in the presence of inhibiting antibodies against TF (10H10) or FVII (3G12) compared to a control IgG.

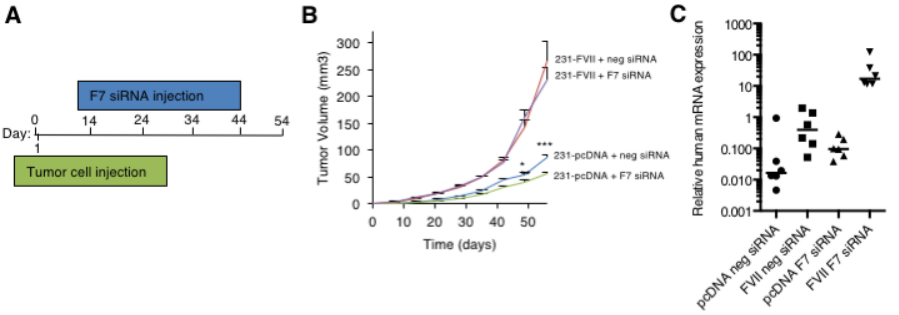
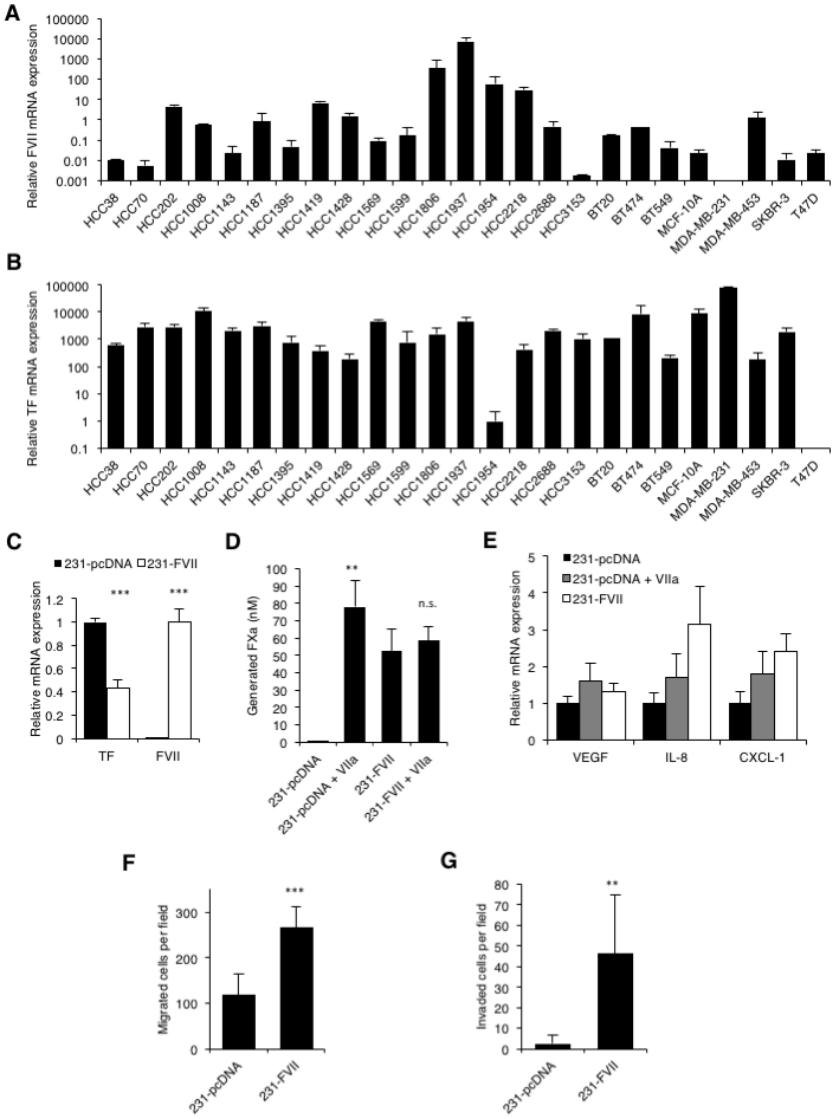
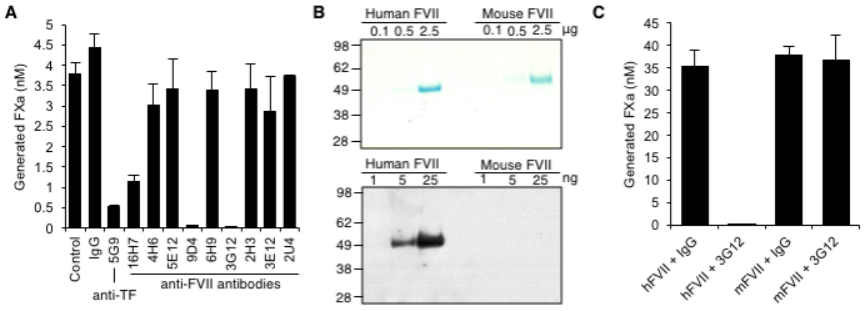


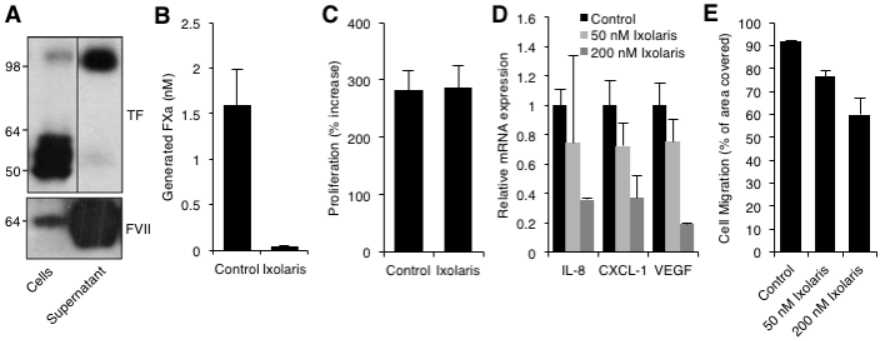
Figure 5. Ectopic FVII-driven tumor growth is independent of circulation FVII. **A** Schematic timeline: F7 siRNA was injected on day 0, followed by subsequent injections. Tumor cells were grafted on day 1, and tumor volume was measured over time. **B** 231-FVII tumor growth was not affected by knockdown of liver F7 expression, while 231-pcDNA tumor development was dependent on circulation FVII levels. **C** Liver metastasis was increased in 231-FVII bearing animals as measured by tumor derived liver mRNA levels. F7 siRNA injection lead to an overall increase in liver metastasis.



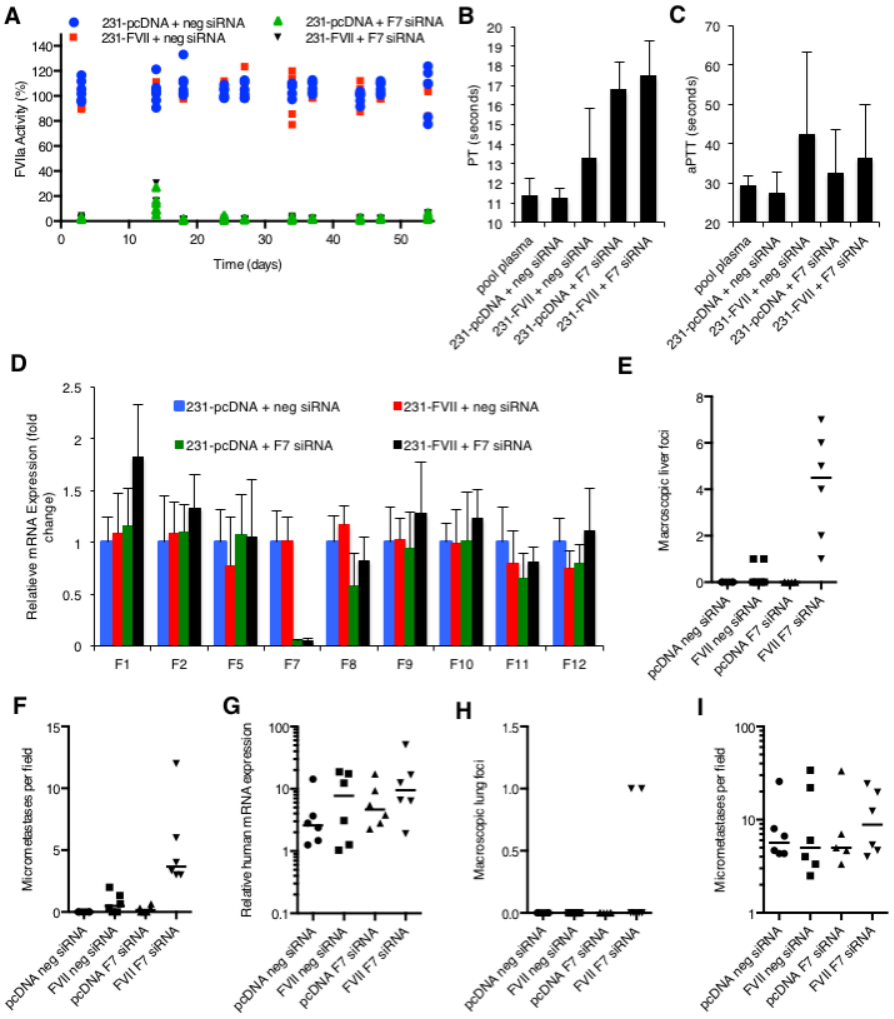
Supplemental Figure 1. FVII and TF expression in breast cancer cell lines. **A** Relative FVII mRNA expression in breast cancer cell lines. **B** Relative TF mRNA expression in breast cancer cell lines. **C** TF and FVII expression in MDA-MB-231 cells transfected with FVII cDNA (231-FVII) or empty vector control (231-pcDNA) was confirmed at the mRNA level. Interestingly, TF expression is halved when FVII is expressed. **D** 231-pcDNA cells required 1 nM recombinant FVIIa to generate FXa, while the potential of 231-FVII cells to generate FXa was not influenced by exposure to FVIIa. **E** mRNA expression of VEGF, IL-8 anf CXCL-1 in 231-pcDNA, 231-pcDNA exposed to 10 nM FVIIa, and 231-FVII cells. **F** Transwell migration and invasion (**G**) was significantly increased in 231-FVII cells compared to 231-pcDNA.



Supplemental Figure 2. **A** Generated FXa was measured: Relipidated TF (Innovin) was exposed to 1 nM FVIIa and 100 nM Fxa after 15 minute incubation with 50 µg/ml antibody. FVII antibodies 16H7, 9D4 and 3G12 significantly inhibited FXa generation. **B** Coomassie staining and Western blot of human and mouse recombinant FVII. 3G12 could detect human but not mouse FVII on Western blot. **C** MDA-MB-231 cells were exposed to 50 µg/ml 3G12 or control IgG for 15 minutes, after which cells were exposed to 100 nM FX and 1 nM human or mouse FVII. Generated FXa was measured after 30 minutes. 3G12 inhibited human but not mouse FVII activity.



Supplemental Figure 3. **A** HCC1937 cells express functional TF and FVII. **B** TF:FVIIa inhibitor Ixolaris inhibits FXa generation on HCC1938 cells. **C** Cell proliferation of HCC1937 is unaffected by Ixolaris. **C** Ixolaris exposure lead to a dose-dependent decrease of expression of IL-8, CXCL-1 and VEGF in HCC1937. **E** Cell migration of HCC1937 was dose-dependently decreased by Ixolaris.



Supplemental figure 4. **A** Plasma FVIIa activity measured throughout the experiment showed effective FVII silencing after F7 siRNA treatment. **B** F7 siRNA treatment prolonged Pro-thrombin (PT) time whereas negative siRNA did not, compared to pool plasma. **C** Activated partial Thromboplastin time (aPTT) was unaffected by siRNA treatment compared to pool plasma. **D** relative gene expression of coagulation factors in mouse liver mRNA at the end of the experiment. Liver metastasis after siRNA treatment was quantified by macroscopic liver foci (**E**) and microscopic cytokeratin$^+$ liver foci (**F**). Lung metastasis was quantified by relative human GAPDH mRNA levels (**G**), macroscopic lung foci (**H**) and microscopic cytokeratin$^+$ foci (**I**). **B-D:** error bar depicts standard deviation **E-I:** horizontal bar depicts median.

Chapter 6 - Tissue Factor associates with survival and regulates tumor progression in osteosarcoma

Tieken C, Verboom MC, Ruf W, Gelderblom H, Bovée JVMG, Reitsma PH, Cleton-Jansen A-M, Versteeg HH.

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Abstract

Osteosarcoma is the most common primary malignant bone tumor. Patients often develop lung metastasis and have a poor prognosis despite extensive chemotherapy and surgical resections. Tissue Factor is associated with poor clinical outcome in a wide range of cancer types, and promotes angiogenesis and metastasis. The role of Tissue Factor in OS tumorigenesis is unknown.

53 osteosarcoma pre-treatment biopsies and four osteosarcoma cell lines were evaluated for Tissue Factor expression, and a possible association with clinical parameters was investigated. Tissue Factor function was inhibited in an osteosarcoma cell line (143B) by shRNA knockdown or specific antibodies, and pro-tumorigenic gene expression, proliferation, matrigel invasion and transwell migration was examined. 143B cells were implanted in mice in the presence of Tissue Factor-blocking antibodies, and tumor volume, micro-vessel density and metastases in the lung were evaluated.

Tissue Factor was highly expressed in 73.6% of osteosarcoma biopsies, and expression associated significantly with disease-free survival. Tissue Factor was expressed in all four investigated cell lines. Tissue Factor was knocked down in 143B cells, which led to reduced expression of *IL-8*, *CXCL-1*, *SNAIL* and *MMP2*, but not *MMP9*. Tissue Factor knockdown or inhibition with antibodies reduced matrigel invasion. Tissue Factor antibodies limited 143B tumor growth *in vivo*, and resulted in decreased intra-tumoral micro-vessel density. Furthermore, lung metastasis from the primary tumor was significantly reduced. Thus, Tissue Factor expression in osteosarcoma reduces metastasis-free survival in patients, and increases pro-tumorigenic behavior both *in vitro* and *in vivo*.

Keywords: osteosarcoma, coagulation, tumor, migration.

Introduction

High-grade conventional osteosarcoma (OS) is the most common primary malignant bone tumor, mostly affecting adolescents. OS is often located in the metaphysis of long bones, usually in the distal femur, proximal tibia

and proximal humerus, and less frequently in the pelvis, spine or skull.(1) The introduction of multi-agent chemotherapy has improved three-year survival from 20% to 60-70%, but no further improvements have been made in the last few decades.(2) OS is highly metastatic and 30-40 percent of all patients develop lung metastases mostly within 2-3 years. Resected tumors are analyzed for chemotherapy-induced necrosis,(1,3) which is a predictor of survival in patients.(4) But metastatic disease at diagnosis, the site and size of the tumor also associate with survival.(1) A suitable prognostic marker for disease progression that can be determined in pretreatment biopsies would be more preferable as this allows for tailored treatment at an earlier time point.

Tissue Factor (TF) is a trans-membrane glycoprotein, functioning as a hemostatic envelope for blood vessels and tissues.(5) Upon tissue damage, TF is exposed to the bloodstream and binds to the plasma protein Factor VII (FVII) to initiate coagulation. FVII is activated upon binding, leading to Factor X (FX) activation by the TF:FVIIa complex. This ultimately leads to prothrombin activation, fibrin deposition, platelet activation and clot formation.
TF is often up-regulated on the surface of tumor cells. In colorectal cancer, mutation of the *K-RAS* oncogene and the loss of p53 results in TF up-regulation via MAPK and PI3K pathways.(6) In glioma cells, overexpression of the oncogenic epidermal growth factor receptor *EGFRvIII* and loss of PTEN function enhance TF expression, again via MAPK and PI3K pathways.(7) TF expression has been associated with metastasis in pancreatic(8), colorectal(9) and lung(10) cancer.

Apart from coagulation activation, TF can activate cell signaling pathways via TF:FVIIa-dependent protease activated receptor 2 (PAR-2) activation. This leads to MAPK and PI3K activation(11), as well as production of pro-angiogenic factors like *VEGF*, *IL-8*, *CXCL-1* and *CTGF*.(12,13) Both the Akt and MAPK pathways have been implicated in OS cell signaling,(14–16) and mutations in *TP53* were recently shown to occur in the majority of OS.(17)

In 2011, Ichikawa *et al.*(18) reported an osteosarcoma patient with a tumor-associated thrombus, suggesting that OS cells locally activate the coagulation cascade. Interestingly, the primary tumor and tumor cells within the associated thrombus were both positive for TF. The same report showed that the OS cell lines MG63, SAOS-2, TE85 and 143B were able to induce thrombin generation, which is suggestive of functional TF expression. In another study, the OS cell line SAOS-2 was shown to express TF, and the TF:FVIIa complex on these cells induced calcium signaling through PAR-2.(19)

Whether TF is widely expressed in OS and directly influences tumor progression has not been reported. Here we show that TF is expressed in primary OS tumors and associates with metastasis-free survival. Inhibition of TF reduces the aggressive phenotype of OS cells *in vitro*, and diminishes experimental tumor growth and metastasis *in vivo*. These findings indicate that TF is a promising target for adjuvant treatment of OS, and a potential predictor of disease progression.

Methods

Patient Material

Formalin-fixed, paraffin-embedded pre-treatment biopsy tissues were collected from high-grade osteosarcoma patients diagnosed between 1984 and 2003; patients with detectable metastasis at the time of diagnosis were excluded from further analyses. Metastasis-free and overall survival was recorded to determine disease progression over time. All patients were treated with doxorubicin and cisplatinum according to a standard protocol in a clinical trial. Human tissue was handled in a coded fashion, according to Dutch national ethical guidelines ("Code for Proper Secondary Use of Human Tissue," Dutch Federation of Medical Scientific Societies). The tissue micro array was constructed as described before.(20) TF protein expression was determined in 53 biopsies using a specific TF antibody

(4503, American Diagnostica, Stamford, CT, USA) by immunohistochemical staining. In short, slides were deparaffinized, rehydrated, after which endogenous peroxidase activity was blocked with 0.3% H_2O_2. Antigen retrieval was performed using sodium citrate buffer for 10 minutes at 100 °C. Sections were blocked with 5% bovine serum albumin in phosphate-buffered saline and incubated overnight at room temperature with 1:125 diluted primary antibody. Sections were incubated with Envision (Dako, Glostrup, Denmark) for 1 hour, visualized with DAB and counterstained with hematoxylin. This antibody does not recognize the soluble alternatively spliced isoform of TF.(21) TF staining was assessed by two independent observers (JB and CT); the kappa inter-observer agreement was 0.881. TF protein expression was scored as staining intensity (0 = negative, 1 = weak, 2 = positive, 3 = highly positive) plus percentage of positive tumor cells (1 = 0-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%) resulting in a combined score between 0 and 7. A score below 4 was considered low TF, 4 or higher was considered as high TF. Chemotherapy induced tumor necrosis of >90% was considered as a good histological response (WHO Classification of Tumors of soft tissue and Bone). Statistical analyses were performed using the statistical software package SPSS (version 21, SPSS Inc., Chicago, IL, USA).

Cell Culture

The cell lines U2OS, SAOS-2, MNNG-HOS, 143B and MCF-7 were purchased from the ATCC (Manassas, VA, USA), the MDA-MB-231mfp cell line was described before(22). Cells were cultured in RPMI1640 (Invitrogen, Karlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin. OS Cell line authentication was confirmed by STR profiling using the GenePrint 10 system (Promega, Madison, WI, USA). TF expression was downregulated in 143B cells by specific TF shRNAs (Sigma Mission library, St. Louis, MO, USA) introduced by lentiviral transduction.

PCR

flTF and asTF mRNA expression was determined using a common TF forward primer and specific asTF and flTF reverse primers. Real-time quantitative PCR was performed using SYBR green (Life Technologies, Carlsbad, CA, USA). Primers are described in Supplementary table 1. To determine the presence of human tumor cells in the lungs of mice, mRNA was isolated from snap-frozen lung material and converted to cDNA, a qPCR using mouse *β-actin* and human *GAPDH* housekeeping genes was used to quantify the presence of human mRNA as a measure for lung metastasis.

In Vitro Assays

FXa generation was measured by seeding $3x10^4$ cells in a 48-well plate, after attaching overnight, the cells were exposed to 100 nM FX (Kordia, The Netherlands) and 1 nM FVIIa (Novo Nordisk, Malov, Denmark) and FXa levels were quantified using the substrate Xa Spectrozyme (American Diagnostica, Greenwich, CT, USA) at 405 nm. Cell proliferation was determined by a methyl-tetrazolium bromide mitochondrial activity (MTT) assay. Anoikis was determined by seeding $1x10^5$ cells in low-adhesion plates and comparing viability on day 0 and 3 using MTT. For cell survival, $1x10^5$ cells were seeded overnight in 24-wells tissue culture plates, washed, and placed on serum-free medium. Cell viability was determined on day 3 using MTT. Cells were exposed to either 50 μg/ml 10H10, 5G9 or IgG control antibody, 10 nM FVIIa, or 100 nM FX and 10 nM FVIIa.
Invasion and migration assays were performed using BD Biocoat Matrigel invasion chambers according to the manufacturer's instructions (BD Biosciences, Palo Alto, CA, USA). In short, $3x10^4$ cells were added to the upper compartment in the presence of 50 μg/ml antibody and allowed to invade for 18 hours at 37°C. Membranes were stained with crystal violet and invaded cells were quantified. Western blots were performed by lysing cells in sample buffer (Invitrogen, Karlsbad, CA, USA), after which lysates were run on 6-18% gradient gels and transferred to PVDF membranes. Membranes were blocked using 5% milk powder and incubated with the

primary antibodies, followed by HRP-conjugated secondary antibodies. Bands were visualized using Western Lightning ECL (PerkinElmer).

In vivo tumor growth assay

$1x10^6$ 143B cells were mixed with 500 μg 10H10, 5G9 (mouse monoclonal antibodies against TF) or a control mouse IgG in PBS and injected subcutaneously (n=6) in NOD-SCID mice (Jackson Laboratories, Westgrove, PA, USA). Tumor growth was measured over time using calipers; tumor volume was calculated as $width^2$ * length * 0.5. Tumors were harvested after 3 weeks and formalin-fixed, paraffin-embedded for further analysis. Lungs were snap-frozen in liquid nitrogen. Animal experiments were approved by the animal ethical committee of the Leiden University Medical Center. Immunohistochemsirty was performed as described above, a vWF antibody (1:5000, DAKO, Glostrup, Denmark) was used to stain microvessels. Ki67 (1:800, DAKO, Glostrup, Denmark) was used to proliferating cells. Ki67 positive nuclei were quantified as described by Tuominen *et al.*(23) Microvessel density was quantified by counting vWF+ microvessels in at least two fields per tumor at 10x magnification. Means were calculated, a t-test was performed to determine statistical significance between IgG control and 10H10 or 5G9 groups.

Results

TF expression is associated with metastasis free survival in osteosarcoma patients

The expression of TF was investigated in a panel of 53 OS biopsies high-grade OS patients without detectable metastasis at the time of diagnosis. All biopsy specimens were positive for TF by immunohistochemistry (IHC), although immuno-reactivity differed widely between samples. Fourteen patient samples were considered low TF (example shown in Fig. 1A) and 39 samples high TF (Fig.1B), as determined by the IHC score. Age, sex, and median follow up were not statistically different between the TF low and TF high groups, as shown in Table 1. Clinical or biological heterogeneity

within the patient group was limited as most patients suffered from conventional OS (supplemental table 2). Patients with high TF more often had a poor histological response to pre-operative chemotherapy compared to patients in the low TF group (odds ratio: 2.21, 95% CI: 0.65-7.52), but this effect did not reach statistical significance. High TF in biopsies significantly associated with reduced metastasis-free survival (Fig. 1C, p=0.042). As metastatic spread in OS severely diminishes overall survival in OS, high TF expression also associated with decreased overall survival, although this does not reach statistical significance (Fig. 1D, p=0.102). As shown in Table 1, the odds ratio of developing metastatic disease upon high tumor TF expression was 5.70 (95% CI 1.12-28.90), whereas the odds-ratio for overall survival during follow-up was 3.75 (95% CI 0.73-19.14).

TF expression by OS cell lines

In order to establish an *in vitro* cell model to study TF-driven tumor progression expression of TF was investigated in OS cell lines. Full length (fl)TF, alternatively spliced (as)TF and PAR-2 expression was examined by PCR and compared to flTF, asTF and PAR2 levels in two breast cancer cell lines with low and high expression levels of these gene products. The OS cell lines U2OS, SAOS-2, MNNG-HOS and 143B expressed similar levels of full length TF as the breast cancer cell line MDA-MB-231mfp. Expression of asTF, a soluble TF isoform implicated in integrin-dependent tumor cell proliferation, ranged from low to undetectable (Fig. 2A). All OS cell lines expressed PAR-2.

The OS 143B cell line was selected for subsequent experiments because of its aggressive phenotype and ability to metastasize in murine models.(24) Total TF expression could be down regulated in OS 143B cells using an shRNA approach. This resulted in a 70% reduction of TF mRNA compared to a scrambled shRNA (Fig. 2B) and similarly reduced TF protein levels determined by Western blot (Fig. 2C). We subsequently observed a 4-fold decrease in the ability to generate FXa after exposure to FVIIa and FX,

showing that TF on OS 143B cells was active, and mRNA reduction led to reduced TF pro-coagulant activity (Fig. 2D).

TF knockdown *in vitro* modulates pro-tumorigenic gene expression but not proliferation

Rapid tumor cell proliferation is a hallmark of cancer progression, and OS 143B is a highly proliferative cell line, both *in vitro* and *in vivo*.(25) We found that cell proliferation was not dependent on TF expression levels determined by a MTT proliferation assay (Fig. 3A). Apart from its role in coagulation activation, TF is also able to activate pro-angiogenic pathways by signaling via PAR-2.(26) We observed that knockdown of TF significantly reduced the expression of the cytokines *IL-8* and *CXCL-1* (Figure 3B), that are both downstream pro-angiogenic effectors of PAR-2 signaling and well-established readouts of TF signaling.(13,27)

Expression of the zinc finger transcription factor Snail has recently been linked to OS cell invasion and migration.(28) We found that TF knockdown led to a significant decrease in *SNAIL* expression in OS 143B cells (Fig. 3B). An essential step for metastasis to occur is the breakdown by matrix metalloproteinases (MMPs) of the extracellular matrix surrounding the primary tumor, and expression of *MMP2* and *MMP9* was previously shown to regulate OS cell invasion and migration.(15,29) We observed that knockdown of TF reduces the expression of *MMP2*, but *MMP9* expression remained unchanged (Fig. 3B).

To determine that the effects of TF knockdown were not caused by off-target effects of the TF shRNA, we used two different shRNAs directed against TF. As shown in Fig. 3D, TF shRNAs 2 and 3 reduced TF mRNA levels by 47.1% and 39.4% respectively, which was confirmed by Western blot (Fig. 3C). This lead to a similar reduction of *IL-8* expression (Fig. 3D), showing that *IL-8* expression is indeed dependent on TF expression levels.

TF inhibition reduces cell migration and invasion

Although metastasis is a multi-faceted process that is difficult to study *in vitro*, the ability of tumor cells to migrate and invade artificial extra-cellular matrices is an established readout for metastatic behavior. Matrigel invasion was 2.3-fold reduced after TF knockdown in OS 143B cells, trans-well migration was 4.1-fold reduced (Fig. 4A). Next TF function in OS was targeted using antibody-mediated blockade specific to TF. The monoclonal TF antibodies 10H10 and 5G9 are specific inhibitors of distinct processes in TF biology; 10H10 inhibits TF:FVIIa dependent PAR-2 activation while 5G9 inhibits FX activation via the TF:FVIIa complex.(30) The TF:FVIIa:FXa complex can also activate PAR-2 as well as trigger subsequent coagulation activation. Matrigel invasion was significantly reduced in OS 143B cells in the presence of the TF antibodies 10H10 or 5G9 compared to a non-specific mouse IgG (Fig. 4B). This inhibiting effect was not explained by 10H10 and 5G9-dependent reduction in proliferation or cell survival, as these antibodies did not substantially influence proliferation or loss-of-anchorage-induced apoptosis (anoikis) (Sup. Fig. 1A and B) in serum-containing media, although addition of high amounts of FVIIa, alone or in combination with FX, could inhibit serum starvation-induced apoptosis (Sup. Fig. 1C). To exclude that this effect was specific for OS 143B cells, we also determined matrigel invasion in the OS cell lines U2OS, SAOS-2 and MNNG-HOS, and observed that the TF antibodies 10H10 and 5G9 both significantly reduced matrigel invasion (Fig. 4C).

Inhibition of TF by specific antibodies reduces tumor growth and lung metastasis

We next evaluated whether TF inhibition can attenuate OS tumor development *in vivo*. OS 143B cells were injected in the presence of the TF antibodies 5G9, 10H10 or an isotype-matched control IgG1 in NOD-SCID mice. The TF antibodies 5G9 and 10H10 antibodies significantly reduced tumor volume over time (Fig. 5A). TF does not regulate OS cell proliferation *in vitro*, thus we reasoned that tumor growth is likely dependent on TF-driven angiogenesis. Tumor sections stained for the proliferation marker

Ki67 did not show a significantly difference in the percentage of proliferating tumor cells when tumors were treated with the TF antibodies 10H10, 5G9 or control IgG (Fig. 5B and 5C). In contrast, micro-vessel density in tumors treated with TF antibodies 5G9 and 10H10 was significantly decreased (Figure 5D and E). Thus, inhibition of TF reduces tumor growth by attenuating angiogenesis. The OS 143B cell line is capable of metastasizing from the primary tumor to distant sites in murine models.(24) By comparing human to mouse housekeeping-gene expression in the lungs we quantified the presence of tumor cells, and observed a more than 200-fold reduction in mice treated with TF antibodies (Fig 5F).

Discussion

This study shows that TF is expressed in primary osteosarcoma, and associated with metastasis-free survival based on pre-treatment biopsy material. Prognosis of disease progression is important for both the patient and treating physician to determine the course of treatment. Treatment usually consists of pre-operative chemotherapy after the diagnosis has been established based on histological examination of a biopsy. Several studies have shown that a good histological response to pre-operative chemotherapy associates favorably with survival.(4,20) This also allows patients with a poor response to chemotherapy to receive alternative chemotherapy regimens, which may improve survival.(31)

Recently, more efforts have been put into identifying adequate predictors of OS progression based on biopsies. A meta-analysis showed that *VEGF* associated with overall and disease-free survival(32). It is interesting to note that *VEGF* expression has been associated with TF expression in lung(33), colorectal(34) and prostate(35) cancer. Increased microRNA-9 expression associated with survival(36), while low expression of microRNAs 183(37) and 223(38) combined with upregulation of Ezrin or Ect2 respectively associated with both metastasis and poor response. The loss of expression of the tumor suppressor p16 associated with decreased survival in patients.(20) *HER2* expression and *TP53* mutations associate with disease

progression in multiple cancer types, but they were not associated with OS progression.(17,39) We have shown here that high expression of TF in OS biopsies associates with decreased metastasis-free survival. It should be noted that the confidence interval was wide (1.12-28.90) despite the fact that most of the patients suffered from conventional OS (Sup. Table 2). Rather, as osteosarcoma is a rare tumor type, we believe the limited number of included patients explains the wide confidence interval. Despite this, we believe that the association between TF and metastasis-free survival provides a rationale for optimized treatment decisions based on TF expression in biopsy material, which is considerably earlier collected than resected material after neo-adjuvant chemotherapy and surgery.

Besides associations with clinical parameters it was also shown that specific inhibition of TF reduces the pro-tumorigenic behavior of OS cells in both *in vitro* and *in vivo* experimental models. Experimental data by others shows that TF drives pro-tumorigenic cellular processes, although this is mostly shown in tumor models of epithelial origin.(26) In this paper we have shown that TF inhibition also reduces angiogenesis and metastatic behavior in tumor cells of mesenchymal origin.

We show here that knockdown of TF expression by shRNAs reduces the expression of pro-angiogenic factors *IL-8* and *CXCL-1 in vitro*, and TF antibodies reduced micro-vessel density in OS tumors *in vivo.* Although angiogenesis is a crucial process for tumor development, a higher micro-vessel density in OS biopsies was associated with more favorable survival rates and a good response to chemotherapy.(40) This paradox is likely caused by better access of chemotherapeutic agents to the tumor cells via the more densely organized intra-tumoral vascular network. We also note that despite reduced micro-vessel density as a consequence of co-injection of tumor cells with TF antibodies did not reduce the number of proliferating cells. Although this appears contradicting, coagulation factor FVII, alone or in combination with FX, inhibits osteosarcoma tumor cell apoptosis. Thus, 10H10 or 5G9 treatment may inhibit this cell survival in vivo, and Ki67

positivity is the resultant of equilibrium between proliferation and cell death. In support, a reduction in vascular density while maintaining Ki67 positivity in vivo that was comparable to control, was also demonstrated after knockout of tumor cell PAR2, the functional TF/FVII receptor.(41) In this study it also remained unclear whether TF induces tumor growth through enhanced vascular density, or whether TF induces tumor growth resulting in more vessel growth. However, as TF is not associated with a proliferative advantage in vitro, we deem the latter scenario unlikely.

Both TF knockdown and inhibition by antibodies reduced the invasiveness of OS cells *in vitro*, and lung metastasis was reduced after TF antibody administration *in vivo*. At present, we believe that the inhibitory action of our TF inhibitory antibodies may be ascribed to TF's role in invasion, rather than its role in fibrin/platelet plug formation on the surface of cancer cells which protects the metastatic cell from the immune system and shear stress.(42) The reason for this is that 10H10 primarily interferes with PAR2 activation rather than TF coagulant function and clot formation (30). We also note that 10H10 could be an interesting approach to targeting TF-driven metastasis in OS. Indeed, clinical use of TF antibodies could be complicated by possible bleeding complications caused by their anticoagulant effects. A clinical trial using a monoclonal TF antibody reported no major bleeding, although dose-dependent minor bleeding was observed.(43)

While this study directly proves that TF dictates OS cell behavior *in vitro* and in an *in vivo* model of OS, it also has a number of limitations. Although it is known that tumor cell grafting in an orthotopic setting results in tumor growth and dissemination that is different from that observed after subcutaneous grafting, we decided to use the subcutaneous model, based on three considerations; i) subcutaneous growth allows more accurate tumor volume measurements in time that grafting in bone, ii) orthotopic graftment, but not subcutaneous grafting, often leads to onset of humane endpoints before detection of wide-spread tumor cell dissemination,(44)

hampering measurement of metastasis, and iii) xenografts from subcutaneously and orthotopically grown OS tumor cells show similar patterns of protein expression and similar histology reminiscent of primary human osteosarcoma.(24)

A second limitation is the limited availability of spontaneously metastasizing OS cell lines(24) which precluded us from confirming the role of TF in OS progression *in vivo* using other cell lines. Nevertheless, as TF antibodies incubated with three other OS cell lines consistently inhibited invasion, a prerequisite for metastasis, we believe that TF's role in invasion is essential for early metastasis.

Finally, our work did not show how TF drives OS progression. It is known that serum contains sufficient levels of FVII to activate PAR2,(45) and a role for 10H10 in inhibiting TF-dependent PAR2 activation is consistent with this view.(30) However, 10H10 also disrupts TF-integrin 1 complexes,(30) and interplay between TF and integrins was previously shown to regulate cell migration.(46) Furthermore, we believe that MMP2 and Snail are not solely responsible for the effects of TF on migration. In the future, knockdown approaches should enable us to decipher whether TF-dependent migration, invasion and metastasis is dependent on PAR2 activation or TF-integrin β1 complexation.

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Table 1. Association of Tissue Factor with OS patient and tumor characteristics					
Characteristic	**TF low N (%)**	**TF high N (%)**	**Total N (%)**	**p-value**	**Odds Ratio (95% CI)**
Total	14 (100)	39 (100)	53 (100)		
Age at diagnosis, y (SD)	15.5 (6.7)	15.3 (6.6)	15.5 (6.6)	0.37	
Sex, female n (%)	8 (57.1)	18 (46.2)	26 (49.1)	0.49	
Median follow-up, mo (range)	208 (33-348)	142 (6-303)	172 (6-348)	0.09	
Histological response to chemotherapy					
Good (>90% necrosis)	7 (50.0)	11 (28.2)	18 (34.0)		2.21 (0.65-7.52)
Poor (≤90% necrosis)	6 (42.9)	27 (69.2)	33 (62.2)		
Undetermined[a]	1 (7.1)	1 (2.6)	2 (3.8)		
Metastasis during follow-up					
Yes	2 (14.3)	20 (51.3)	21 (39.6)		5.70 (1.12-28.90)
No	12 (85.7)	19 (48.7)	32 (60.4)		
Overall Survival					
Deceased	2 (14.3)	15 (35.9)	17 (32.1)		3.75 (0.73-19.14)
Alive	12 (85.7)	24 (64.1)	36 (67.9)		

[a] Two patients did not receive pre-operative chemotherapy.

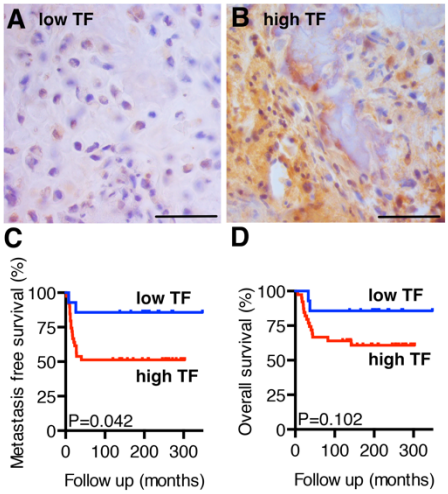


Figure 1. TF in Osteosarcoma biopsies reduces metastasis-free and overall survival. The presence of TF protein was determined by IHC in 69 biopsies. **A** low TF expression and **B** high TF expression in OS biopsies. Bars represent 50 μm. **C** Metastasis free survival is significantly reduced in patients with high TF (p: 0.042, log-rank test). **D** High TF in biopsies reduced overall survival in trend (p: 0.102, log-rank test).

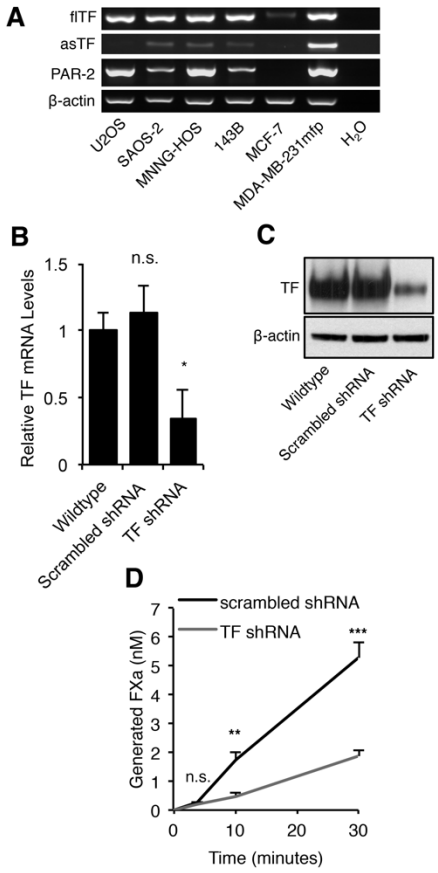


Figure 2. TF is expressed and active on OS cell lines. **A** mRNA expression of flTF, asTF and PAR-2 in OS cancer cell lines. MCF7 and MDA-MB-231mfp were included as a negative and positive control respectively. **B** qPCR and **C** Western blot showing that TF expression is reduced in 143B cells using a TF shRNA. A scrambled shRNA does not alter TF expression. **D** The ability to generate FXa by 143B after exposure to 1 nM FVIIa and 100 nM FX is attenuated because of TF expression knockdown. n.s: not significant, *: $p < 0.05$, $p < 0.01$ ***$p < 0.001$. All values expressed as mean ±SD, Students t-test was used.

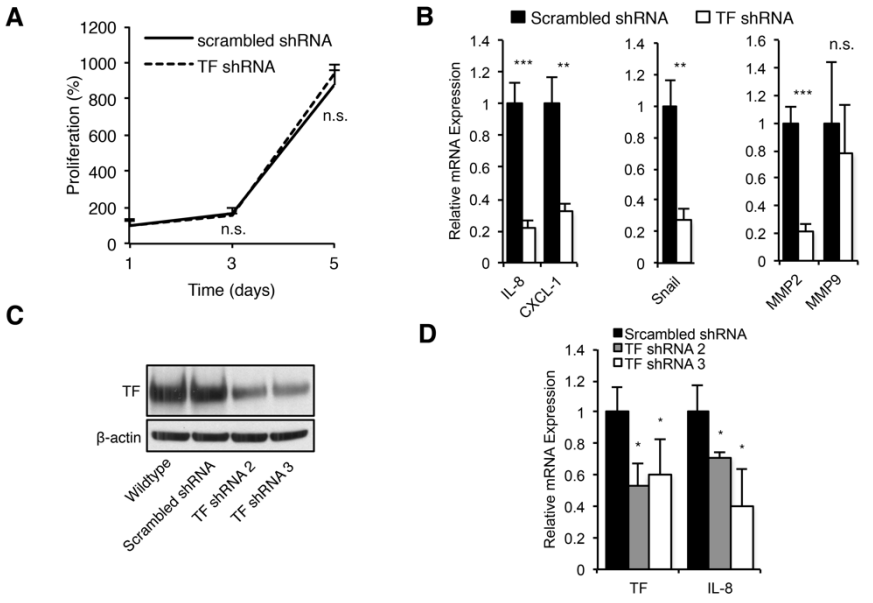


Figure 3. TF knockdown reduces pro-tumorigenic gene expression but not cell proliferation. **A** TF knockdown did not alter 143B cell proliferation by MTT assay. **B** TF knockdown significantly reduced expression of pro-angiogenic factors *IL-8* and *CXCL-1*, the EMT marker *SNAIL*, and *MMP2* but not *MMP9*. **C** TF expression was knocked down in 143B using two alternative TF shRNA construct. **D** TF shRNAs 2 and 3 significantly reduced TF expression, resulting in a dose-dependent IL-8 downregulation *p <0.05 **p <0.01 ***p < 0.001. All values expressed as mean ±SD, Students t-test was used.

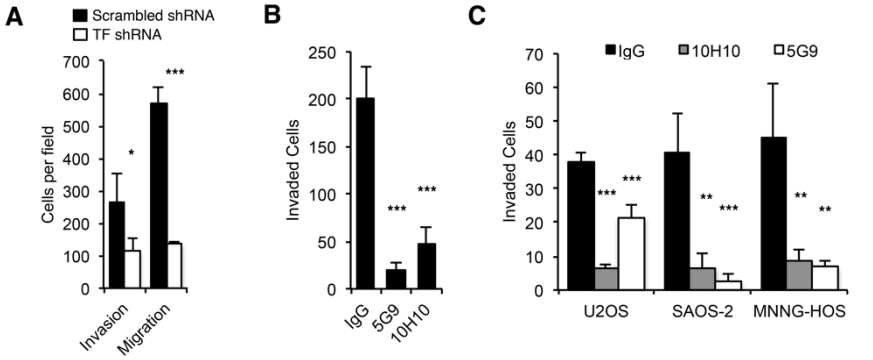


Figure 4. TF inhibition reduces migration and invasion *in vitro*. **A** TF knockdown reduces matrigel invasion and transwell migration. **B** TF antibodies 5G9 and 10H10 significantly reduce matrigel invasion. **C** Matrigel invasion is reduced by both TF antibodies 5G9 and 10H10 in U2OS, SAOS-2 and MNNG-HOS. *p <0.05 **p <0.01 ***p < 0.001. All values expressed as mean ±SD, Students t-test was used.

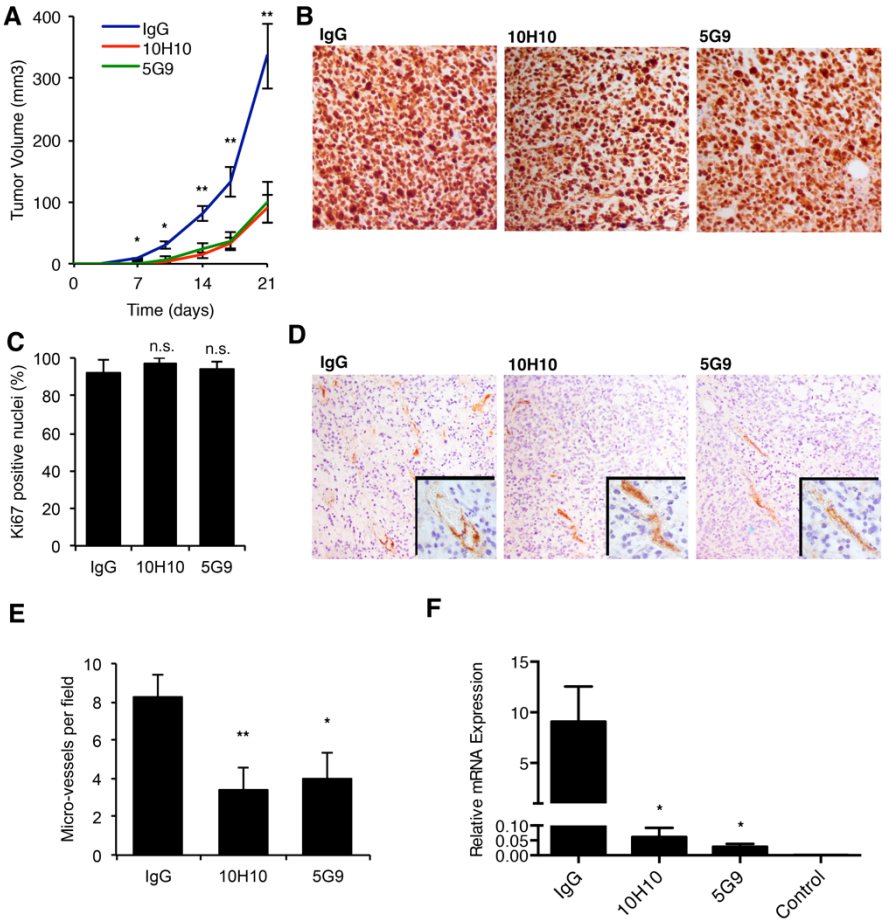
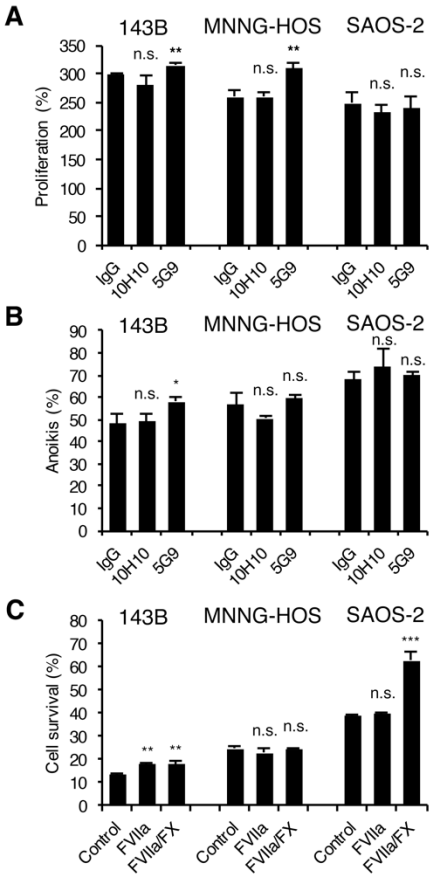


Figure 5. TF antibodies inhibit OS tumor growth and metastasis *in vivo*. **A**143B Cells mixed with 5G9, 10H10 or a nonspecific IgG control were subcutaneously injected in NOD-SCID mice. Both 10H10 and 5G9 significantly reduced tumor volume. **B** Representative Ki67 staining of tumor sections **C** The percentage of Ki67 positive nuclei were quantified per tumor field **D** Tumors were analyzed by IHC to asses vascular density (vWF), inset shows a high magnification of a vWF^{+} micro-vessel. **E** Quantification of micro-vessels per field. **F** qPCR on lung cDNA comparing human GAPDH to mouse β-actin to quantify the metastatic load. Control represents lung cDNA from a mouse not exposed to tumor cells. Error bar = SEM. n.s: not significant *p <0.05 **p <0.01

Supplementary table 1: PCR Primers		
	Forward (5' - 3')	Reverse (5'-3')
TF	TTACACAACAGACACAGAGTGTGA	
flTF		GAATATTTCTTTCCTGAACTTGAAG
asTF		TTGAACACTGAAACAGTAGTTTTCTCC
PAR-2	TGGATGAGTTTTCTGCATCTGTCC	CGTGATGTTCAGGGCAGGAATG
β-actin	CAAGAGATGGCCACGGCTGCT	TCCTTCTGCATCCTGTCGGCA
Quantitative PCR primers		
GAPDH	TTGCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG
TF	CTGCTCGGCTGGGTCTTC	AATTATATGCTGCCACAGTATTTGTAGTG
IL-8	AGGTGCAGTTTTGCCAAGGA	TTTCTGTGTTGGCGCAGTGT
CXCL-1	AGTCATAGCCACACTCAAGAATGG	GATGCAGGATTGAGGCAAGC
MMP-2	TGACGGTAAGGACGGACTC	ATACTTCACACGGACCACTTG
MMP-9	CTGGGCAGATTCCAAACCT	TACACGCGAGTGAAGGTGAG
Snail	CCCCAATCGGAAGCCTAACT	GCTGGAAGGTAAACTCTGGATTAGA
mouse β-actin	ACCGTGAAAAGATGACCCAGATC	TAGTTTCATGGATGCCACAGG

Supplementary Table 2. Tissue Factor expression in OS histological subtypes				
	TF low N (%)	**TF high N (%)**	**Total N (%)**	**p-value**
Total	17 (100)	52 (100)	69 (100)	
Histological subtype				
Conventional	9 (52.9)	36 (69.2)	45 (65.2)	
Chondroblastic	2 (11.8)	3 (5.8)	5 (7.2)	
Fibroblastic	1 (5.9)	2 (3.8)	3 (4.3)	
MFH-like	1 (5.9)	2 (3.8)	3 (4.3)	
Teleangiectatic	1 (5.9)	1 (1.9)	2 (2.9)	
Partially chondroblastic	0 (0.0)	2 (3.8)	2 (2.9)	
Small cell	1 (5.9)	0 (0.0)	1 (1.4)	
Metachronous	1 (5.9)	0 (0.0)	1 (1.4)	0.46
Partly teleangiectatic, partly small cell	1 (5.9)	0 (0.0)	1 (1.4)	
Anaplastic	0 (0.0)	1 (1.9)	1 (1.4)	
Partly sclerotic	0 (0.0)	1 (1.9)	1 (1.4)	
Partly teleangiectatic	0 (0.0)	1 (1.9)	1 (1.4)	
High grade surface	0 (0.0)	1 (1.9)	1 (1.4)	
Osteoblastic, partly sclerotic	0 (0.0)	1 (1.9)	1 (1.4)	
Undetermined	0 (0.0)	1 (1.9)	1 (1.4)	



Supplemental figure 1. Effect of TF antibodies, FVIIa and FX on proliferation and survival in OS cells. **A** TF antibody 10H10 does not alter OS cell proliferation, while 5G9 confers a slight increase in proliferation in 143B and MNNG-HOS, but not SOAS-2 compared to an IgG control. **B** Loss of anchorage-induced apoptosis (anoikis) is not affected in MNNG-HOS and SOAS-2 cells by either 10H10 or 5G9. In 143B cells, 5G9 but not 10H10 showed a slight increase in anoikis. **C** FVIIa or FVIIa/FX exposure in OS cells has varying effects on serum-starvation-dependent apoptosis. Both FVIIa and FVIIa/FX increase cell survival in 143B cells, but not in MNNG-HOS cells. Cell survival in SOAS-2 cells increased after FVIIa/FX exposure, but not FVIIa alone. n.s: not significant *p <0.05 **p <0.01 ***p < 0.001.

Chapter 7 - Tissue Factor isoform and Factor VII expression and clinical outcome in colorectal cancer

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Manuscript in preparation

Abstract

Introduction: Tissue Factor (TF) is the initiator of coagulation, but also expressed by cancer tissues and a potent modulator of tumor angiogenesis. TF is widely expressed in various solid tumors, and has been associated with tumor stage, grade, and patient survival. Although the role of TF in colorectal tumor progression has been studied before, the individual contribution of TF isoforms full length (fl)TF and alternatively spliced (as)TF have as of yet not been reported. Recent evidence showed that TF's natural ligand Factor VII (FVII) is also expressed in tumor specimens, but its role in colorectal cancer progression has not yet been studies thoroughly. The aim of the present study is to determine flTF, asTF and FVII expression in a cohort of colorectal cancer specimens and investigate clinical outcome.
Methods: tumor specimens of 353 colorectal cancer patients collected between 1991-2001 were stained by immunohistochemistry using specific flTF, asTF and FVII antibodies. Stainings were scored by two independent observers, and associated with clinical parameters and patient survival.
Results and conclusion: High flTF expression was positively associated with high TNM stage, as well as non-mucinous tumors and chemotherapy treatment. High flTF expression reduced survival in right-sided tumors and in patients who did not receive chemotherapy. Both high asTF and FVII expression associated with non-mucinous tumors but clinical outcome was not statistically affected. Both flTF and asTF are widely expression in colorectal cancer, but only high flTF expression appears to have a clear impact on colorectal cancer progression.

Introduction

Tissue Factor (TF), a 47 kDa transmembrane protein, is the primary initiator of blood coagulation and a modulator of (tumor) angiogenesis.[1,2] After blood vessel damage TF expressed on the subendothelium is exposed to Factor VII (FVII) from the circulation. TF and FVII form a binary complex, after which FVII is activated to FVIIa. This TF:FVIIa complex can bind and

activate Factor X (FX), which subsequently leads to thrombin formation and fibrin deposition.[2]
Systemic hypercoagulability is often observed in cancer patients and is associated with tumor progression.[3] In up to 90% of patients with metastatic disease clotting abnormalities are observed, and thrombosis is currently the second-most common cause of cancer-related mortality.[4] Expression of TF in tumor cells and patient specimens has been reported in various types of solid tumors.[1] Expression of TF has been associated with tumor stage and metastasis in colorectal cancer.[5] Furthermore, TF is an independent risk factor for hepatic metastasis in colorectal cancer.[6] TF expression in colorectal cancer cells is driven by activation of the K-ras oncogene and p53 inactivation, and TF inhibition reduces angiogenesis in *in vivo* models.[7] Apart from activation of coagulation, the TF:FVIIa complex can activate the protease activated receptor (PAR) 2 on the surface of tumor cells, which leads to increased expression of pro-angiogenic factors like vascular endothelial growth factor (VEGF), IL-8 and CXCL-1.[1] In a panel of colorectal cancer patients TF expression was positively associated with both VEGF staining and increased micro-vessel density.[8]

In 2003, a novel splice variant of TF, alternatively spliced TF (asTF) was described. asTF lacks exon 5, which encodes the transmembrane domain of full length TF (flTF), rendering asTF soluble. Furthermore, because of a frame shift in the TF mRNA, asTF contains a unique C-terminal tail.[9] Whether asTF contributes to coagulation is debated[10–12], but several studies provide evidence for a role of asTF in angiogenesis and tumor proliferation: asTF promotes angiogenesis via integrin ligation, independently of FVIIa or PAR-2 in experimental models.[13] Furthermore, asTF promotes *in vivo* tumor growth and micro-vessel density in both pancreatic[14] and breast[15] cancer models. Whether asTF is expressed in colorectal cancer and contributes to disease progression is currently unknown.

More recently, expression of FVII by tumor cells has been reported in a number of studies. FVII mRNA is expressed in ovary, gastric and breast

cancer cell lines.[16] Furthermore, FVII expression increased in ovarian cancer cells under hypoxic conditions[17] and FVII expression enhanced tumor cell migration.[16] Furthermore, in a small panel of colorectal cancer specimens FVII expression was associated with both tumor stage and liver metastasis.[18]

So far, studies that focused on the expression of TF and its association with clinical outcome in colorectal cancer have not differentiated between the full length and alternatively spliced isoforms of TF. In this study we have determined the expression of flTF, asTF and FVII in a panel of colorectal cancer patient specimens and investigated their respective contribution to disease outcome and survival.

Methods

Patient cohort

The patient population comprises 470 colorectal cancer patients treated in the Leiden University Medical Centre. All patients received surgery for their primary tumor between 1991 and 2001. Tumor specimens, clinical-pathological data and follow up were collected. Patients with a history of cancer other than basal cell carcinoma or cervical carcinoma *in situ*, patients with more than one colorectal tumor, or patients who received radio- or chemotherapy before tumor resection were excluded from the analyses (n = 117), the remaining cohort contained 353 colorectal cancer patients.

Immunohistochemistry

Tissue sections of 4 μm were cut from a tissue micro array containing formalin-fixed paraffin-embedded colon cancer specimens. Tumor specimens from each patient were included in triplicate. Immunohistochemical staining was performed by standard procedures. Briefly, sections were deparaffinized and rehydrated, after which endogenous peroxidase activity was blocked with 0.3% H_2O_2. For flTF and FVII staining, antigen retrieval was performed in sodium citrate buffer for 10 minutes at 100 °C. Sections were blocked for 1 hour in 10% fetal bovine

serum in 1% PBS/BSA, after which sections were incubated overnight with primary antibody in 1% PBS/BSA. The following primary antibodies and concentrations were used: 4503 (flTF, 1:100, American Diagnostica, Stamford, CT, USA), Rabmab1 (asTF, 1 μg/ml, rabbit monoclonal antibody specific for asTF as described before[15]) and N3C3 (FVII, 1:800, Genetex, Irvine, CA, USA) Sections were washed with PBS, followed by incubation with Envision (Dako, Glostrup, Denmark) for 1 hour, visualized with DAB and counterstained with hematoxylin.

Assesment of flTF, asTF and FVII protein expression

All tumor specimens were scored by CT, a subset of the specimens was scored by BÜ and a kappa inter-observer variability of 0.834 was calculated. flTF and asTF staining was scored by percentage of positive tumor cells per tumor specimen. High flTF expression was considered >50% positive tumor cells, resulting in 219 specimens with high flTF expression and 75 with low expression. asTF positive tumor percentage was split in quartiles, where the lowest quartile was considered low asTF expression, quartiles 2-4 were considered high asTF. This resulted in 65 low asTF and 205 high asTF tumor specimens. FVII was scored by staining intensity, as follows: 0 = negative staining, 1 = slightly positive staining, 2 = positive staining, 3 = highly positive staining. An intensity of 0-1 was considered low expression, staining intensity of >1 was considered high expression. This resulted in 60 low FVII and 237 high FVII expression specimens.

Statistical analysis

Statistical analyses were performed using the SPSS statistical software package (version 21. SPSS Inc., Chicago, IL, USA). The Chi-square test was used to calculate association between protein expression and clinical parameters. The Kaplan-Meier method was used to create relapse-free and overall survival curves; the log-rank test was used to determine statistical significance when comparing survival curves. A p-value below 0.05 was considered statistically significant.

Results

High flTF expression associates with tumor stage and patient survival

Hundred and seventy-two tumor specimens presented with high flTF expression, while 122 specimens showed low flTF expression (examples shown in Fig. 1A). High flTF expression was positively associated with a higher TNM stage ($p = 0.002$, Table 1). Patients suffering from tumors with high flTF expression more often presented with non-mucinous adenocarcinoma compared to low flTF expression (91.3% vs. 76.2% respectively, $p < 0.001$). Furthermore, patients with tumors showing high flTF expression more often received chemotherapy (19.8% vs. 10.7%, $p = 0.036$). Age, gender, differentiation, microsatellite stability and location of the primary tumor were not statistically different.

High flTF expression was associated with reduced disease free and overall survival compared to low flTF expression (Fig 2A,B), although this did not reach statistical significance ($p = 0.166$ and $p = 0.071$ respectively). Interestingly, for patients that did not receive chemotherapy, both disease-free and overall survival was significantly reduced when high flTF expression was observed (Fig. 2C and 2D, $p = 0.024$ and $p = 0.043$ respectively). Furthermore, patients that presented with a primary tumor located on the right side, disease-free and overall survival in patients with high flTF expression was also significantly reduced (Fig. 2E and 2F, $p = 0.026$ and $p = 0.021$ respectively).

asTF associates with mucinous aspect but not with tumor stage

Whether asTF is widely expressed in colorectal cancer has not been reported yet. In this cohort, 205 patient specimens were found to have high asTF expression (examples pictured in Fig. 1B). Tumors with high asTF expression more often had a non-mucinous adenocarcinoma compared to low asTF expression (Table 1, $p = 0.007$). asTF expression was not significantly associated with other clinical parameters, including tumor stage and differentiation. Patients with high tumoral asTF expression

showed reduced disease-free and overall survival compared to low asTF expression (Fig 3AB), although these effects failed to reach statistical significance (p = 0.050 and 0.081 respectively).

FVII expression is associated with mucinous aspect and microsatellite stability, but not survival

In 237 patient specimens (80.1%) a strong positivity for FVII was observed (Fig. 1C), while the surrounding tissue and normal colorectal mucosa stained negative. As observed with flTF and asTF, high FVII expression was more often present in non-mucinous tumors compared to tumors with low FVII expression (Table 1, 86.1% vs. 73.3% respectively, p = 0.007). Furthermore, high FVII expression associated with microsatellite stability, which is associated with poor survival.[19] 73.0% of patients with high FVII expression had microsatellite stability, compared to 46.7% with low FVII (p = 0.014). FVII expression was not significantly associated with gender, age, stage, differentiation, chemotherapy or location of the tumor. Furthermore, FVII expression had no influence on disease-free or overall survival (Fig. 3B).

Discussion

Although the role of TF expression on patients outcome in colorectal cancer has been reported before,[5,6,20] these studies failed to differentiate between the flTF and asTF isoforms. Here we report that both flTF and asTF are widely expressed in colorectal cancer. High flTF but not asTF expression is associated with higher stage in colorectal cancer, although both are associated with reduced disease-free and overall survival. Interestingly, high flTF expression only led to a significant reduction of survival in patients with a primary tumor on the right side. Tumors located on the right side are more common,[21] present with a more advanced stage, and in stage III patients right-sided tumors showed increased mortality compared to left-sided tumors.[22] Why flTF associates with decreased survival in

colorectal tumors on the right side only and not on the left would be an interesting avenue for further investigation.

Since the discovery of asTF in 2003, its role in coagulation and cancer progression has been a matter of debate. Although some studies point to an involvement of asTF in coagulation,[9,10] others showed no pro-coagulant properties of asTF.[11,12] Whether and how asTF promotes tumor progression has recently become more clear: in pancreatic cancer asTF mRNA is expressed in tumor cell lines[23] and promotes *in vivo* tumor growth and angiogenesis.[14] AsTF has pro-angiogenic potential via a different mechanism than flTF; by ligation of specific β1 and β3 integrins but independently of FVIIa and PAR2.[13] Our group has shown that asTF is widely expressed in breast cancer, associated with tumor stage and promotes *in vivo* tumor growth via β1 integrins.[15] This report is the first to provide evidence of widespread asTF expression in a large colorectal cancer cohort. Although no association with TNM stage was observed, a clear decrease in survival for up to 200 months was observed. As these data do not offer a complete picture on the contribution of asTF in colorectal cancer progression, more research is needed to fully elucidate its pro-oncogenic properties.

Recent evidence of ectopic FVII expression in cancer tissues[18] and cell lines[16,17] provides a novel insight in the role that the extrinsic coagulation factors can play in tumor progression. In this colorectal cancer cohort, FVII expression does not associate with survival in patients. A recent study showed that FVII protein expression was associated with tumor stage[18], but such a finding was not replicated in this (6-fold larger) cohort. The underlying mechanism why FVII expression shows different effects on clinical outcome in different solid tumor types is as of yet not understood, and warrants future research.

It is interesting to note that flTF, asTF and FVII were more often highly expressed in non-mucinous tumors. Whether the presence of mucin influences clinical outcome and is associated with stage remains a controversial topic. A recent study demonstrated increased recurrence and

a reduced rate of survival in non-mucinous adenocarcinoma compared to mucinous tumors.[24] While a 2012 meta-analysis found a decrease in survival in mucinous versus non-mucinous tumors, 3 out of 4 of their included studies reported improved survival for mucinous adenocarcinoma when combined with micro-satellite instability.[25] It is interesting to note that although high FVII expression showed no influence on patient survival, high FVII expression is positively associated with both non-mucinous tumor morphology and microsatellite stability.

In summary, in this first study to differentiate between TF isoform expression in colorectal cancer specimens. FlTF but not asTF expression was associated with tumor stage and diminished survival, although both are widely expressed. Although high FVII expression is also widespread in this cohort, an association with clinical outcome was lacking.

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Table I. Patient Characteristics of the Total Colon Cancer Cohort and stratified for FVII, fITF and asTF										
	Total	fITF –	fITF +	p	asTF -	asTF +	p	FVII -	FVII +	p
	(n=353)	(N=122)	(N=172)		(n=65)	(N=205)		(n=60)	(N=237)	
Gender (%)										
Male	174 (49.3)	57 (46.7)	91 (52.9)	0.296	33 (50.8%)	101 (49.3%)	0.833	28 (46.7)	123 (51.9)	0.469
Female	179 (50.7)	65 (53.3)	81 (47.1)		32 (49.2%)	104 (50.7%)		32 (53.3)	114 (48.1)	
Age in years (%)										
Below 65	148 (41.9)	49 (40.2)	73 (42.4)	0.696	24 (36.9%)	89 (43.4%)	0.355	24 (40.0)	97 (40.9)	0.896
Above 65	205 (58.1)	73 (59.8)	99 (57.6)		41 (63.1%)	116 (56.6%)		36 (60.0)	140 (59.1)	
TNM stage (%)										
1	68 (19.3)	33 (27.0)	19 (11.0)		13 (20.0%)	28 (13.7%)		11 (18.3)	41 (17.3)	
2	133 (37.7)	45 (36.9)	63 (36.6)		26 (40.0%)	77 (37.6%)		26 (43.3)	84 (35.4)	
3	92 (26.1)	25 (20.5)	56 (32.6)	0.002	13 (20.0%)	63 (30.7%)	0.325	17 (28.3)	66 (27.8)	0.434
4	53 (15.0)	18 (14.8)	32 (18.6)		12 (18.5%)	35 (17.1%)		6 (10.0)	43 (18.1)	
Missing	7 (2.0)	1 (0.8)	2 (1.2)		1 (1.5%)	2 (1.0%)			3 (1.3)	
Differentiation (%)										
Moderate	183 (51.8)	55 (45.1)	98 (57.0)		37 (56.9%)	105 (51.2%)		25 (41.7)	127 (53.6)	
Poor	27 (7.6)	11 (9.0)	14 (8.1)	0.722	4 (6.2%)	21 (10.2%)	0.147	7 (11.7)	18 (7.6)	0.294
Good	67(19.0)	21 (17.2)	33 (19.2)		6 (9.2%)	39 (19.0%)		8 (13.3)	48 (20.3)	
Missing	76 (21.5)	35 (28.7)	27 (15.7)		18 (27.7%)	40 (19.5%)		20 (33.3)	44 (18.6)	
Mucinous aspect(%)										
No	291 (82.4)	93 (76.2)	157 (91.3)		48 (73.8%)	178 (86.8%)		44 (73.3)	204 (86.1)	
Fully	38 (10.8)	20 (16.4)	6 (3.5)	<0.001	12 (18.5%)	12 (5.9%)	0.007	13 (21.7)	18 (7.6)	0.007
Partly	17(4.8)	8 (6.6)	6 (3.5)		4 (6.2%)	11 (5.4%)		3 (5.0)	10 (4.2)	
Missing	7 (2.0)	1 (0.8)	3 (1.7)		1 (1.5%)	4 (2.0%)			5 (2.1)	
Microsatellite stability(%)										
MSS	215 (60.9)	83 (68.0)	120 (69.8)	0.208	42 (64.6%)	146 (71.2%)	0.466	28 (46.7)	173 (73.0)	0.014
MSI	35 (9.9)	9 (7.4)	22 (12.8)		8 (12.3%)	20 (9.8%)		10 (16.7	22 (9.3)	
Missing	103 (29.2)	30 (17.4)	30 (17.4)		15 (23.1%)	39 (19.0%)		22 (36.7)	42 (17.7)	

Chemotherapy (%)										
No	298 (84.4)	109 (89.30	138 (80.2)	0.036	54 (83.1%)	169 (82.4%)	0.906	52 (86.7)	197 (83.1)	0.505
Yes	55 (15.6)	13 (10.7)	34 (19.8)		11 (16.9%)	36 (17.6%)		8 (13.3)	40 (16.9)	
Location (%)										
Left	210 (59.5)	78 (63.9)	107 (62.2)	0.508	41 (63.1%)	132 (64.4%)	0.993	32 (53.3)	155 (65.4)	0.156
Right	110 (31.2)	33 (27.0)	54 (31.4)		19 (29.2%)	61 (29.8%)		21 (35.0)	65 (27.4)	
Missing	33 (9.3)	11 (9.0)	11 (6.4)		5 (7.7%)	12 (5.9%)		7 (11.7)	17 (7.2)	

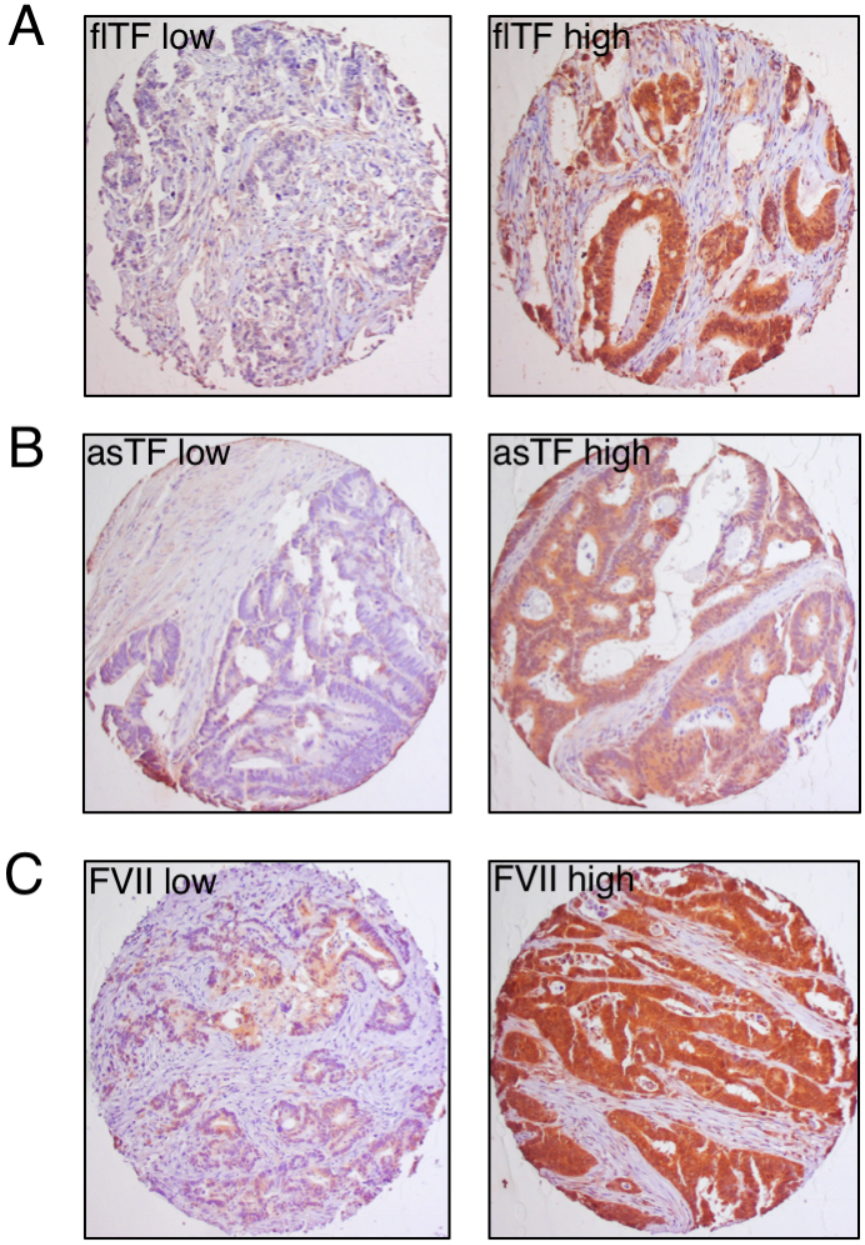

Figure 1. Representative photos of colorectal cancer tissue specimens. **A** low and high flTF expression. **B** low and high asTF expression. **C** low and high FVII expression. All panels depict a 10x magnification of a tissue micro array tumor specimen. Brown color depicts positive staining.

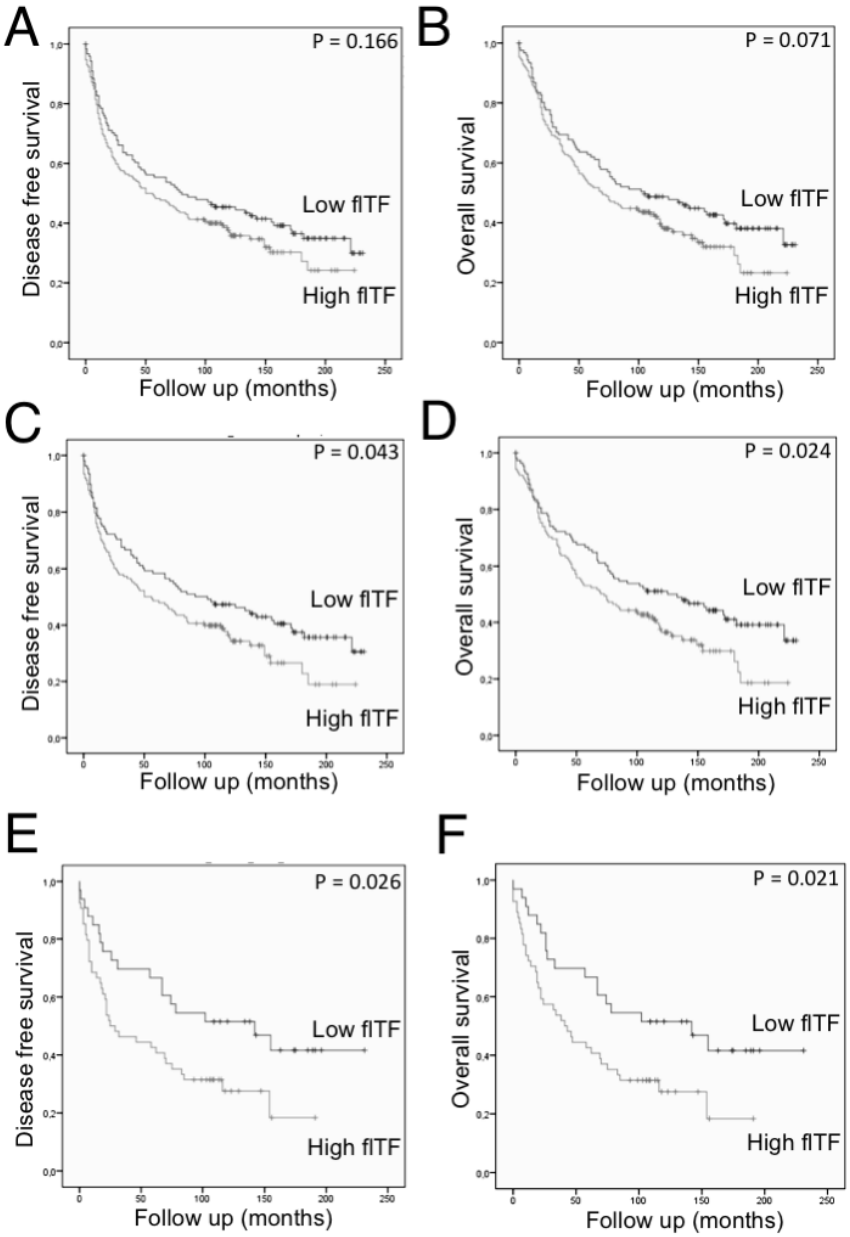

Figure 2. Kaplan-Meier survival analysis of flTF expression in colorectal cancer. **A,B** Expression of flTF and the association with disease free and overall survival. **C,D** flTF expression associated with survival in patients that did not receive chemotherapy. **E,F** flTF expression associated with survival in patient with a tumor located on the right side. Log-rank p values are shown in each graph. Vertical dashes depict censored data.

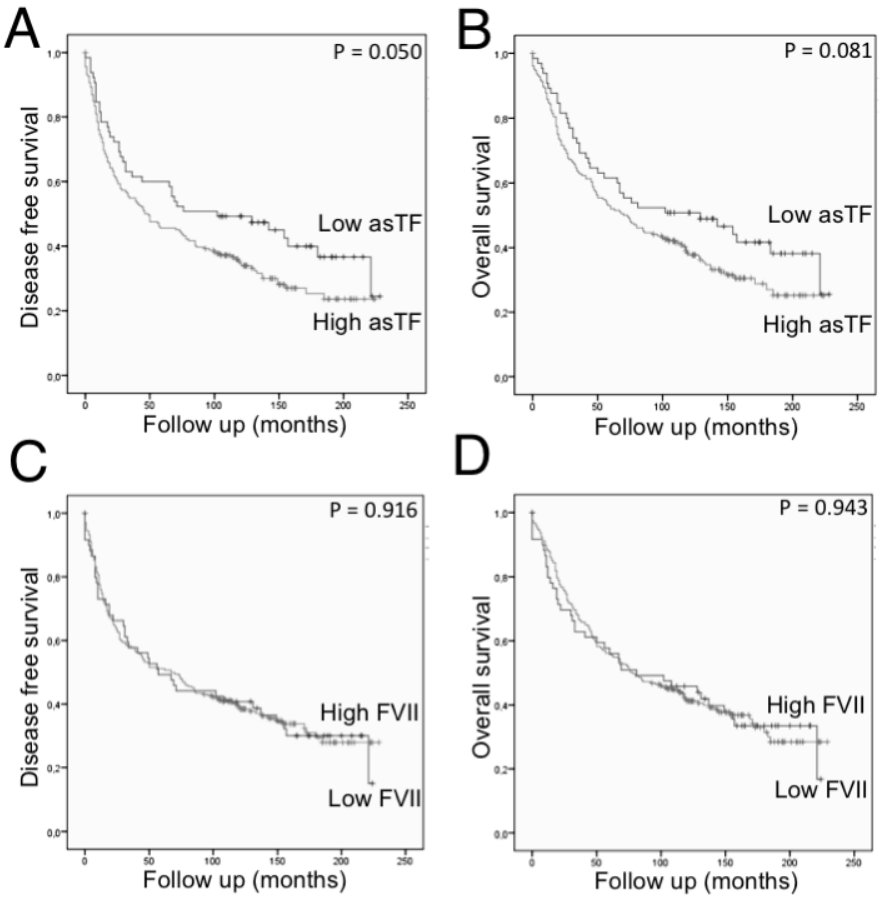

Figure 3. Kaplan-Meier survival analysis of asTF and FVII expression in colorectal cancer. **A,B** asTF expression associates with reduced disease-free and overall survival. **C,D** FVII expression does not associate with reduced disease-free and overall survival. Log-rank p values are shown in each graph. Vertical dashes depicts censored data.

Chapter 8 – General discussion and summary

Background

The human body contains a complex blood coagulation system that ensures that blood remains fluid in the circulation enabling transport of oxygen, nutrients and immune cells. Whereas a disruption of the vascular wall immediately triggers the formation of a local blood clot to prevent excessive bleeding. This precarious balance is directly regulated by full length Tissue Factor (flTF), the main initiator of extrinsic coagulation and focal point of this thesis. Whereas most coagulation factors are synthesized in the liver and circulate in the bloodstream, flTF is constitutively expressed on the surface of adventitial cells surrounding blood vessels.[1] Vessel wall disruption exposes flTF to Factor VII (FVII) from the circulation, which is activated to FVIIa upon binding. This flTF:FVIIa complex binds and activates Factor X (FX), which ultimately leads to thrombin formation and a stable fibrin clot.[2]

Abnormal activation of the coagulation cascade can ultimately lead to thrombosis, which is often observed in cancer patients.[3] In recent decades it has become clear that tumor cells display aberrant expression of coagulation factors, in particular flTF. Tumor-expressed flTF is able to activate protease activated receptors (PARs), promoting both angiogenesis and tumour cell migration.[4,5] In 2003 alternatively spliced Tissue Factor (asTF) was identified; the transmembrane domain of the TF pre-mRNA transcript is skipped leading to a soluble protein with a C-terminal tail distinct from that of flTF as a results of a frameshift.[6] Although no clear role for asTF in blood coagulation has been identified yet, asTF expression has been observed in several solid tumor tissues and is implicated in cancer progression.[7,8] A large part of this thesis focuses on the distinct properties of asTF compared to flTF in cancer biology and clinical outcome.
Recently the ectopic expression of FVII in tumor cell lines and specimens has been observed.[9,10] Whereas FVII is normally synthesized in the liver and present in the circulation, FVII expression by tumor cells can be triggered by hypoxia.[11] Studies described in this thesis point towards associations between FVII expression and patient survival in breast and colorectal

cancer. These studies also present evidence how ectopic FVII expression promotes tumor progression by augmenting flTF non-haemostatic signalling in breast cancer.

Full length Tissue Factor associated with decreased survival and drives tumor angiogenesis and metastasis

Ever since the 1990's the expression of Tissue Factor (TF) by tumor cells has been associated with cancer progression in various solid tumor types like breast[12], lung[13], colorectal[14], pancreatic[15] and liver.[16] The discovery of asTF raises the issue whether reported associations between what was assumed to be flTF expression and clinical parameters in cancer patient are confounded by asTF expression. As the functional role of asTF in tumor biology is distinct from flTF (described in **chapter 3**) it is imperative to clearly distinguish between both isoforms to unravel their respective contribution to clinical outcome in cancer patients.

In this thesis a strict distinction was made between flTF and asTF, using both *in vitro* expression systems for either flTF or asTF, as well as antibodies specific to only one of the isoforms. In **chapter 3** we report that flTF expression in breast cancer was associated with a higher tumor grade. The study described in **Chapter 6** is the first report to show that flTF is widely expressed in osteosarcoma, and high flTF expression is associated with diminished survival. In **chapter 7** we present the first report distinguishing between flTF and asTF expression in a colorectal cancer cohort: flTF was associated with tumor stage, and high flTF expression associated with diminished survival in patients with a right-sided primary tumor. In summary, high flTF expression is a clear indicator of diminished survival in a multitude of solid cancer types.

The mechanism of non-hemostatic signaling by flTF in cancer cells has been thoroughly investigated in the last years. In 2004 the deletion of the flTF cytoplasmic domain was shown to enhance experimental tumor growth and angiogenesis.[17] In fact, the cytoplasmic domain acts as a negative

regulator of protease activated receptor (PAR) 2-dependent signaling, while phosphorylation of the cytoplasmic domain abolishes this inhibition.[18] In turn the flTF:FVIIa induced PAR2 activation releases suppression of α3β1 integrins[19] and upregulates pro-angiogenic IL-8 expression.[20] Furthermore, flTF is constitutively associated with β1 integrins on tumor cells facilitating flTF-dependent PAR2 activation;[5] this crosstalk between flTF and β1 integrins has been established as a potent mechanism for cell migration (Figure 1).[21] In 2008 Versteeg *et al.* characterized monoclonal flTF antibodies 5G9 and 10H10: whereas 5G9 inhibited FXa generation by the flTF:FVIIa complex, 10H10 inhibited β1 interaction, PAR2 activation by the flTF:FVIIa complex and subsequently experimental tumor growth and angiogenesis in a MDA-MB-231mfp breast cancer xenograft model.[5] As 10H10 did not inhibit flTF:FVIIa-dependent FX activation, this allowed for the inhibition of either flTF's pro-coagulant (via 5G9) or non-hemostatic (10H10) properties (Figure 1). In **chapter 3** 10H10 did not inhibit *in vitro* proliferation of MDA-MB-231mfp cells, suggesting that flTF non-hemostatic signaling does not necessarily enhance tumor growth by promoting cell proliferation but rather via angiogenisis.[5] Interestingly, in **chapter 6** we showed that both 5G9 and 10H10 were both equally potent in inhibiting cell invasion and *in vivo* tumor growth and lung metastasis in osteosarcoma models. Similarly, both 5G9 and 10H10 inhibited M24met (melanoma) tumor growth *in vivo*.[5] This may be explained by the fact that, while 5G9 does not inhibit binary TF:FVIIa signaling, it does inhibit the flTF:FVIIa:FXa ternary complex, which is also capable of activating PAR2. Furthermore, the TF:FVIIa:FXa complex can also activate PAR1, promoting cell migration.[22] Variance of expression levels of PAR1 and PAR2 in different tumor types can explain the different effects of 5G9 and 10H10 inhibition of tumor growth. Ultimately, it shows that flTF-driven tumor growth is not yet completely understood.

Alternatively spliced Tissue Factor as a potent driver of tumor progression via β1 integrin ligation

The identification of asTF fueled an immediate search as to whether asTF has a functional role in blood coagulation and if so, how this possible function was different from flTF. Bogdanov *et al*. suggested in their initial report that asTF may be involved in hemostasis: they found that asTF circulates in human plasma, co-localizes with platelets, is present in human thrombi and reduces clotting time.[6] Endothelial cells were shown to synthesize and release asTF after TNF-α and IL-6 stimulation, increasing the pro-coagulability of the supernatant.[23] These finding were disputed by Censarek *et al*.[24] who reported that asTF overexpressed in HEK293 cells could not induce FXa generation or thrombin formation. Similarly, Hobbs *et al.* found that flTF but not asTF overexpressed in a pancreatic cancer cell lines reduces clotting time.[8] In 2006 murine asTF was characterized, but only contained 3% pro-coagulant activity when compared to murine flTF.[25] Although this topic remains controversial, no evidence that asTF promotes coagulation in the absence of flTF has been presented yet.

A MIA PaCa-2 pancreatic cancer cell xenograft model showed that asTF overexpression significantly increases *in vivo* tumor expansion compared to both flTF overexpression and vector control.[8] Furthermore, tumors expressing asTF presented with increased vascular leakage, intra-tumoral blood vessels and proliferating ($PCNAP^+$) tumor cells. In 2009 Van den Berg *et al.* reported that recombinant asTF increases angiogenesis similar to VEGF both in *in vivo* and *ex vivo* models.[7] Interestingly, this was not dependent on either FVIIa or PAR2, but rather on ligation of β1 and β3 integrin subsets (Figure 2). Similarly, overexpression of asTF in the murine cardiomyocyte-like HL-1 cell line induced pro-angiogenic properties.[26] As asTF appears to function independently of FVIIa and PAR2, we set out in **chapter 3** to determine how asTF and flTF contribute differentially in breast cancer biology. AsTF but not flTF overexpression in MCF-7 breast cancer cells significantly increased *in vitro* cell proliferation as well as expression of a more pro-oncogenic genotype. AsTF was released in the supernatant and

also enhanced proliferation in empty vector transfected MCF-7 cells when exposed to conditioned medium from asTF expressing cells, suggesting a paracrine mode of action (Figure 2). Furthermore, β1 integrin antibody blockade significantly reduced cell proliferation in asTF-expressing cells, and inhibited asTF:β1 integrin interaction as shown by co-immunoprecipitation and collagen I binding assays. In **chapter 4** we observed that the asTF-induced proliferative advantage was dependent on the presence of estradiol (E2), pointing to an asTF/E2 synergy possibly due to shared downstream signaling targets.
Overexpression of asTF in MCF-7 cells led to increased tumor expansion *in vivo* compared to cells transfected with an empty vector control, whereas flTF overexpression did not (**chapter 3**). Similar to what was observed by Hobbs *et al.* tumoral micro-vessel density and the number of proliferating cells were significantly increased when asTF was overexpressed. Enhanced tumor expansion by asTF overexpression was greatly enhanced when estrogen-releasing pellets were placed subcutaneously (**chapter 4**), again pointing to an asTF/ER signaling pathway synergy in breast cancer cells.

As asTF and flTF are often co-expressed in tumor cells, we set out to establish the unique role of asTF-driven tumor progression in the presence of flTF. The MDA-MB-231mfp cell line was derived from MDA-MB-231 cells xenografted in the mammary fat pad of immuno-deficient SCID mice, yielding a more aggressive breast cancer cell line.[27] MDA-MB-231mfp cells expressed abundant levels of both TF isoforms, and β1 integrin-specific blocking antibodies, as well as asTF-specific antibodies could inhibit cell proliferation. Tumor expansion was attenuated by asTF blockade, and a reduction of the number of proliferation tumor cells was observed, while micro vessel density remained unaffected.

Expression of asTF in cancer and clinical outcome

In **chapter 3** we presented the first large study to report abundant asTF in breast cancer specimens: asTF was present in >95% of tumor specimens (n=574), whereas healthy mammary tissue stained positive for asTF in 4% of

stained tissues. High asTF expression associated with a higher histological grade and tumor size. AsTF expression was not associated with estrogen receptor (ER) status, but in **chapter 4** we did observe clear overlap between asTF dependent gene expression profiles in breast cancer cells and ER-dependent gene regulation. When we re-evaluated the associations between asTF expression and clinical parameters by stratifying tumor specimens for ER status, we observed an association between asTF expression and tumor grade and size in ER^+ but not ER^- tumors. As flTF expression was associated with tumor grade in both ER^+ and ER^- tumors, this finding suggested that asTF, but not flTF, cooperates with the ER signaling pathway in breast cancer.

In **chapter 7** we report abundant asTF expression in a large colorectal cancer cohort (n=353). Although no association with tumor grade was observed, high asTF expression associated with a borderline-significant decrease in disease-free survival, (p=0.050). AsTF expression was associated with non-mucinous tumors, which is associated with increased recurrence and diminished survival.[28]

Tumor-derived Factor VII drives tumor progression via the flTF:FVIIa:PAR2 complex

The role of flTF in both coagulation and oncogenic processes has been widely established, relying on the formation of the flTF:FVIIa binary complex on phospholipid surfaces leading to PAR2 activation. Whereas the expression of flTF on the surface of cancer cells has been studied in detail, the source of FVII is assumed to be the blood. Although FVII *is* abundantly present in plasma, the early phases of tumor development are characterized by a lack of access to the circulation, resulting in hypoxic conditions. In 2006, Koizume *et al.* reported that tumor cell lines synthesize FVII ectopically, and that this pool of FVII regulates cancer cell migration and invasion in a flTF-dependent manner.[29] Furthermore, ectopic FVII expression was enhanced under hypoxic conditions, suggesting a role for ectopic FVII tumor progression prior to angiogenesis. Another mode of inducing FVII expression is proposed by Naderi *et al*., who reported co-

expression of the androgen receptor (AR) and FVII in a panel of breast cancer cell lines, and showed that AR activation induced ectopic FVII synthesis and enhanced pro-coagulant activity.[30]

Ectopic FVII expression in cancer specimens has been reported sporadically. In twenty ovarian tumor specimens, FVII was co-expressed with TF.[9] In a colorectal cancer cohort (n=55), FVII was reported on both protein and mRNA level, and associated with liver metastasis.[10] In **chapter 5** we report that 39% of tumor specimens of a large cohort of breast cancer patients (n=574) stained positive for FVII. To exclude that positive FVII staining was caused by FVII taken up by tumor cells from the circulation, we examined FVII mRNA expression in a subset of specimens. FVII protein expression associated with FVII mRNA levels, tumor grade, ER and PgR status, TGF β RII, TGF receptor 1 and 2, and flTF. Furthermore, FVII expression associated with reduced patient survival. This effect on survival was even more pronounced when flTF and FVII were co-expressed, suggesting that the flTF:FVII complex synthesized ectopically by tumor cells furthers disease progression, possibly before the tumor is sufficiently exposed to the circulation that contains ample amounts of liver-derived FVII.

Although we found a clear association between FVII expression and cancer progression in breast cancer in **chapter 5**, this relationship is not as straightforward in colorectal cancer: In **chapter 7** we observed high FVII expression in 80.1% (n=237) of colorectal cancer specimens, but FVII expression was not associated with patient survival or tumor stage. FVII expression was present in 53 out of the 60 (88.3%) specimens with low FVII expression, possibly explaining why no significant effect of FVII expression on clinical outcome was observed. High FVII expression was significantly associated with non-mucinous tumor and micro-satellite stability, a combination associated with reduced survival in a 2012 meta-analysis.[31] Although tumors from various origin have different underlying molecular processes that drive oncogenesis, it is tempting to draw up a general hypothesis as to how the expression of ectopic FVII by tumor cells could

promote tumor progression. Koizume *et al.*[11] show that FVII is synthesized under hypoxic conditions *in vitro*. As early tumor development is characterized by lack of vascularization and thus FVII derived from the circulation, ectopic synthesis may enable flTF:FVII signaling before proper access to the circulation. This could give the tumor a head start in both angiogenesis and metastatic spread that translates to decreased patient survival. As virtually all tumor specimens removed from patients are properly vascularized and thus have ample access to circulation-derived FVII, they might not be the ideal material to study FVII-driven cancer biology on. Furthermore, in our xenograft models tumor size was measured over time using calipers through the skin, which limits the detection and measurement of small tumors. Thus, this technique is not suitable for the measurement of developing tumors where angiogenesis has not yet yielded an intra-tumoral blood vessel network to facilitate exponential tumor expansion. In **chapter 5** we observed that tumor growth of flTF-expressing MDA-MB-231 cells was modestly inhibited when circulation FVII was not present. Although this effect was minimal, this strengthens the hypothesis that both circulation- and tumor-derived FVII can enhance tumor growth, but ectopic FVII expression does supply a higher concentration at an early stage where exposure to the circulation is limited.

Conclusions and future directions

This thesis provides a clear body of evidence that tumoral flTF expression is associated with decreased survival in various solid tumor types. Ectopic expression of FVII in breast tumors significantly decreased survival. Both *in* vitro and *in vivo* experiments show that ectopic FVII expression augments flTF signaling leading to increased tumor cell invasion, tumor growth and metastasis independently of circulation FVII. In summary, ectopic FVII enhanced the effects of flTF signaling in cancer progression, possible before proper angiogenesis has sufficiently connected tumor tissue to the circulation. Another hypothesis is that tumor cells expressing flTF, FVII and PAR2 form functional intra-cellular signaling complexes that result in more enhanced signaling compared to membrane bound flTF:FVIIa signaling via

PAR2 (Figure 1). This could explain the observation in **chapter 5** that MDA-MB-231 cells expressing FVII produce higher mRNA levels of pro-angiogenic cytokines compared to MDA-MB-231 cells exposed to high-dose recombinant FVIIa. Nevertheless, as membrane bound flTF:FVIIa complexes are internalized by cells it is difficult to distinguish between flTF:FVIIa complexes formed intra-cellularly and internalized flTF:FVIIa and this hypothesis may prove difficult to prove.
FlTF and FVII co-expression in breast cancer was associated with diminish survival in breast cancer patients, making flTF/FVII an interesting target for therapeutic intervention. A clinical trial investigating a TF antibody reported no major bleeding, although minor bleeding was reported in a dose-dependent fashion.[32] The monoclonal flTF antibody 10H10 could therefore be a highly interesting clinical intervention to curb flTF signaling, as it is a potent inhibitor of flTF and β1 integrin interaction without inhibiting coagulation activation.

Whether tumoral asTF expression affects patient survival remains unclear. No relation between asTF and survival was observed in breast tumors, although in colorectal cancer patient high asTF expression conferred a lower survival for up to 200 months. AsTF has been established as a potent driver of tumor cell proliferation via a unique subset of integrins, whereas flTF has not been implicated in cell proliferation. Although this thesis provides novel insights in the respective contributions of the TF isoforms flTF and asTF, it is currently unclear whether and if there is any (functional) interaction between flTF and asTF promoting tumor progression. Co-expression of flTF and asTF is often observed in tumor specimens, making it an interesting avenue for future research.

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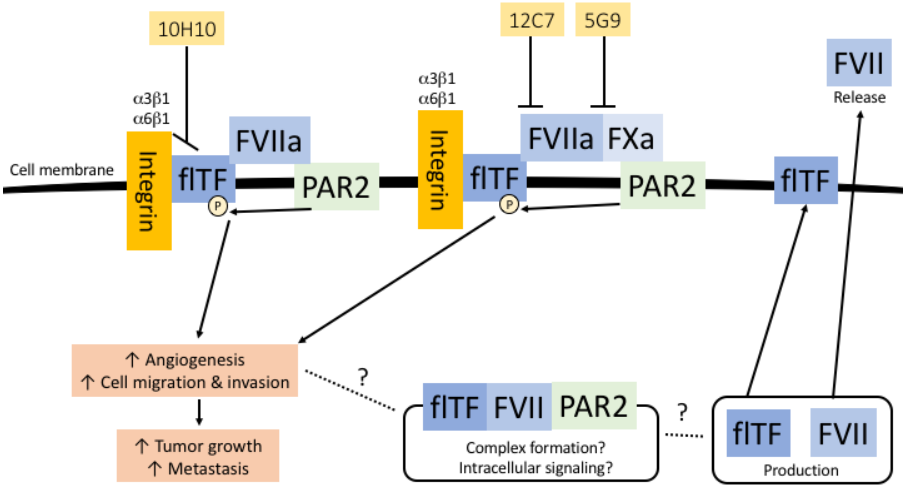

Figure 1. flTF signaling pathway via PAR2 and integrin ligation. The membrane bound flTF/FVIIa and flTF/FVIIa/FXa complex signal via PAR2 when coupled to α3β1 and α6β1 integrins, leadinf to enhanced angiogenesis and tumor cell migration and invasion. The monoclonal flTF antibody 10H10 inhibits flTF-integrin interaction, whereas the flTF antibody 5G9 inhibits the activation of FX to FXa by the flTF:FVIIa complex. 12C7 is an inhibiting mouse monoclonal antibody specific against human FVII(a). flTF and FVII are produced by tumor cells: flTF is incorporated in the cell membrane whereas FVII is released. It is currently unclear whether flTF and FVII produced by tumor cells can form intracellular flTF:FVIIa complexes capable of signaling.

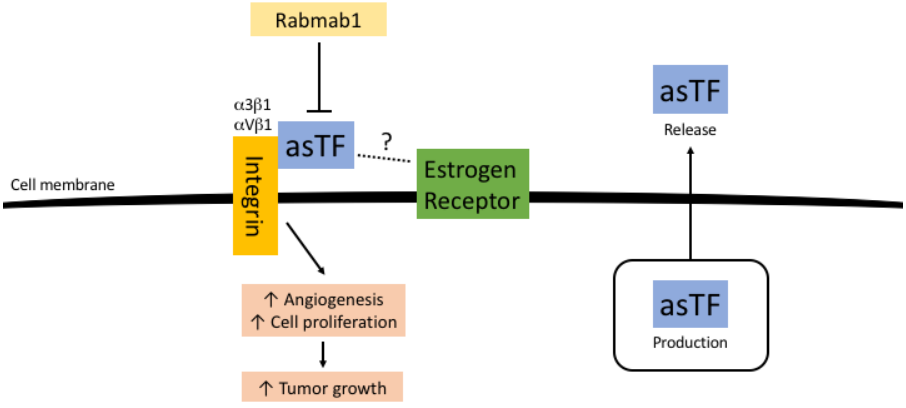

Figure 2. AstF promotes cell proliferation and angiogenesis via ligation of integrin subsets. AsTF is produced and released by tumor cells, where it can promote both cell proliferation and angiogenesis by ligation of α3β1 and αVβ1 membrane bound integrin subsets, leading to increased *in vivo* tumor growth. Signaling the estrogen pathway greatly increases asTF-dependent tumor growth, but the underlying interaction has not yet been identified.

Nederlandse discussie en samenvatting

Achtergrond

Het menselijk lichaam heeft een complex bloedstolling systeem wat zorgt dat bloed in de circulatie vloeibaar blijft en het vervoer van zuurstof, voedingsstoffen en immuun cellen mogelijk maakt. Maar zodra de wand van een bloedvat wordt doorbroken wordt onmiddellijk een lokaal bloedstolsel gevormd om ernstig bloedverlies te stoppen. Deze precaire balans wordt direct gereguleerd door full length Tissue Factor (flTF), de belangrijkste initiator van extrinsieke bloedstolling en het onderwerp van dit proefschrift. Terwijl de meeste bloedstollingsfactoren in de lever worden gemaakt en vrij circuleren in de bloedbaan, wordt flTF constitutief tot expressie gebracht op de oppervlakte van adventitiële cellen die de bloedvaten omringen. Zodra de bloedvatwand doorbroken wordt, komt flTF in aanraking met Factor VII (FVII) uit de circulatie, welke na binding met flTF meteen geactiveerd wordt tot FVIIa. Dit flTF:FVIIa complex kan vervolgens Factor X (FX) binden en activeren, wat uiteindelijk leidt tot trombine vorming en een stabiel bloedstolsel.

Abnormale activatie van de stollingscascade kan leiden tot trombose, wat vaak in kankerpatiënten wordt geobserveerd. In de afgelopen decennia is duidelijk geworden dat tumorcellen stollingsfactoren tot expressie brengen, met name flTF. Dit tumor-flTF is in staat de protease activated receptors (PARs) te activeren, waardoor angiogenese en tumorcel migratie bevorderd wordt. In 2003 is alternatively spliced Tissue Factor (asTF) geïdentificeerd; het transmembraan domein van het TF pr-mRNA transcript wordt overgeslagen, wat leidt tot een oplosbaar eiwit met een C-terminale staart die door een frameshift uniek is vergeleken met flTF. Hoewel er tot nu toe geen duidelijke rol voor asTF in bloedstolling is ontdekt, is asTF expressie wel geobserveerd in verschillende soorten solide tumoren, en speelt mogelijk een rol in kanker progressie. Een groot deel van dit proefschrift richt zich op de unieke eigenschappen van respectievelijk asTF en flTF in kanker biologie en overleving in patiënten.

Recent is geobserveerd dat FVII ectopisch tot expressie komt in tumor cellijnen en monsters. Normaal gesproken wordt FVII gesynthetiseerd in de lever en is aanwezig in de bloedbaan, maar FVII expressie door tumoren

kan ook worden aangedreven door hypoxie. De in dit proefschrift beschreven studies wijzen op associaties tussen FVII expressie en overleving in borst- en darmkanker patiënten. Deze studies presenteren ook bewijs dat ectopische FVII expressie tumor progressie bevordert door non-hemostatische flTF signalering te stimuleren in borstkanker.

Full length Tissue Factor is geassocieerd met verminderde overleving en stuurt tumor angiogenese en metastasering aan

Sinds de jaren '90 is de expressie van Tissue Factor (TF) door tumorcellen geassocieerd met kanker progressie in verschilleden solide kanker zoals borst-, long-, darm-, alvleesklier- en leverkanker. De ontdekking van asTF werpt de vraag op of deze associaties tussen wat werd beschouwd als flTF en klinische parameters in kankerpatiënten niet verward is met asTF expressie. Aangezien de rol van asTF in tumor biologie zich onderscheid van die van flTF (beschreven in hoofdstuk 3) is het noodzakelijk om een strikt onderscheid te maken tussen beide isovormen vanwege hun respectievelijke rol in de overleving van kankerpatiënten.

In dit proefschrift wordt een strikt onderscheid gemaakt tussen flTF en asTF, in zowel *in vitro* expressiesystemen met flTF dan wel asTF, maar ook het gebruik van antilichamen specifiek tegen slechts één van de isovormen. In hoofdstuk 3 rapporteren we dan flTF expressie in borstkanker is geassocieerd met een hogere tumor graad. Hoofdstuk 6 is de eerste studie die laat zien dat flTF tot expressie komt in osteosarcoma, en hoge flTF expressie blijkt geassocieerd met een verminderde overleving. In hoofdstuk 7 presenteren we voor het eerst een studie die onderscheid maakt tussen flTF en asTF in een colorectaal kanker cohort: flTF was geassocieerd met verminderde overleving in patiënten met een tumor aan de rechterzijde. Samengevat is hoge flTF expressie een duidelijke indicator voor verminderde overleving In verscheidende types solide tumoren.
Het mechanisme achter non-hemostatische signalering van flTF in kankercellen is de laatste jaren in detail onderzocht. In 2004 was aangetoond dat de deletie van het cytoplasmatische domein van flTF leidt tot een toename van experimentele tumorgroei en angiogenese. Dit cytoplasmatische domein blijkt als een negatieve regulator van PAR2-afhankelijke signalering te werken, fosforylering van het cytoplasmatische domein verwijdert deze remming. Tegelijkertijd zorgt de door flTF:FVIIa

geïnduceerde PAR2 activatie dat de onderdrukking van α3β1 integrines wegvalt en gaat de pro-angiogene expressie van IL-8 omhoog. flTF is constitutief geassocieerd met β1 integrines op tumorcellen en faciliteert flTF-afhankelijke PAR2 activatie; deze samenspraak tussen flTF en β1 integrines is een potent mechanisme voor cel migratie. In 2008 hebben Versteeg *et al.* de monoclonale flTF antilichamen 5G9 en 10H10 gekarakteriseerd: terwijl 5G9 de FXa generation door het flTF:FVIIa complex blokkeert, remt 10H10 juist β1 integrine interactie en PAR2 activatie door het flTF:FVIIa complex en vervolgens ook experimentele tumorgroei en angiogenese in een MDA-MB-231mfp borstkanker xenograft model. Aangezien 10H10 de activatie van FXa door flTF:FVIIa juist niet remt is het mogelijk om de pro-coagulante (via 5G9) en de non-hemostatische (10H10) eigenschappen van flTF te onderzoeken. In hoofdstuk 3 heeft 10H10 geen effect op *in vitro* proliferatie van MDA-MB-231mfp cellen, wat suggereert dat de non-hemostatische signalering van flTF niet per se tumorgroei bevordert via cel proliferatie maar mogelijk eerder via angiogenese. In hoofdstuk 6 laten we zien dat 5G9 en 10H10 beide even effectief zijn in het remmen van *in vitro* cel invasie én *in vivo* tumorgroei en long metastasering in osteosarcoma modellen. Een vergelijkbare observatie is gedaan in M24met (melanoma) cellen: 5G9 en 10H10 remmen beide tumorgroei *in vivo*. Een mogelijke verklaring is dat 5G9 de signalering van flTF:FVIIa niet remt, maar wel de vorming van het flTF:FVIIa:FXa complex, dat ook in staat is om PAR2 de activeren. Ook is het flTF:FVIIa:FXa complex in staat om PAR1 te activeren, wat ook cel migratie bevordert. Variatie in expressie niveaus van PAR1 en PAR2 in verschillende tumor types zou de verschillende effecten van 5G9 en 10H10 op de remming van tumorgroei mogelijk ook kunnen verklaren. Uiteindelijk is het complete mechanisme achter flTF-gedreven tumorgroei nog niet geheel ontrafeld.

Alternatively spliced Tissue Factor als een potente drijfveer van tumor progressie via β1 integrin ligatie

Door de identificatie van asTF is meteen een zoektocht gestart naar een mogelijk functionele rol voor asTF in bloedstolling, en of deze mogelijke rol afwijkt van flTF. Bogdanov *et al.* suggereerden in hun oorspronkelijke verslag dat asTF mogelijk betrokken is bij hemostase: asTF circuleert in plasma, co-lokaliseert met bloedplaatjes, is aanwezig in humane trombi en vermindert de stollingstijd. Endotheliale cellen blijken asTF te synthetiseren

en scheiden asTF uit na TNF-α of IL-6 stimulatie, waardoor het supernatant meer pro-coagulant wordt. Deze bevindingen werden echter tegengesproken door Censarek *et al*., zij rapporteerden dat asTF, tot overexpressie gebracht in HEK293 cellen, niet in staat was FXa te genereren of trombine te vormen. Hobbs *et al.* vonden dat flTF, maar juist niet asTF, dat tot over-expressie was gebracht in een alvleesklier tumor de stollingstijd kon verkorten. In 2006 is muis-asTF gekarakteriseerd, maar muis-asTF bleek maar 3% stollingsactiviteit te hebben vergeleken met muis-flTF. Al blijft dit onderwerp controversieel, er is tot nu toe geen bewijs geleverd dat asTF is staat is stolling te bevorderen in de afwezigheid van flTF.

Een MIA PaCa-2 alvleesklier tumorcel xenograft model liet zien dat overexpressie van asTF vergelijken met flTF of de vector controle tumor expansie *in vivo* significant vergrootte. Verder bleken tumoren die asTF tot overexpressie brachten meer vasculair te lekken en meer prolifererende ($PCNAP^+$) cellen te bevatten. In 2009 rapporteerden van den Berg *et al*. dat recombinant asTF angiogenese in *in vivo* en *ex vivo* modellen versterkte in vergelijkbare mate als VEGF. Dit effect bleek niet afhankelijk te zijn van FVIIa of PAR2, maar juist van de ligatie van specifieke β1 en β3 integrine subtypes. Verder bleek ook dat asTF overexpressie in de muis cardiomyocyt-achtige HL-1 cellijn pro-angiogene eigenschappen vertoonde. Aangezien asTF onafhankelijk lijkt te functioneren van FVIIa en PAR2, hebben we in hoofdstuk 3 gepoogd uit te zoeken hoe asTF en flTF op verschillende wijze de borstkanker biologie beïnvloed. Overexpressie van asTF (maar niet flTF) in MCF-7 borstkankercellen verhoogde de *in vitro* cel-proliferatie significant en leidde tot een meer pro-oncogeen genotype. AsTF werd vrijgegeven in het supernatant en versterkte ook proliferatie in MCF-7 cellen die met de lege vector getransfecteerd waren als deze cellen werden blootgesteld aan geconditioneerd medium van asTF expressie cellen, wat een paracrine mechanisme suggereert. Verder zorgt antilichaam blokkade van β1 integrine voor verminderde cel-proliferatie van cellen die asTF tot expressie brengen, maar ook verminderde asTF:β1 integrine interactie in co-immunoprecipitatie en collageen I binding studies. In hoofdstuk 4 observeerden we dan asTF-geïnduceerde proliferatie afhankelijk was van de aanwezigheid van estradiol (E2), wat duidt op een asTF/E2 synergie door mogelijk gedeelde signaleringsdoelen.
Overexpressie van asTF in MCF-7 cellen leidde *in vivo* tot toegenomen tumorexpansie vergeleken met cellen getransfecteerd met een lege vector,

terwijl flTF overexpressie dit effect niet liet zien (hoofdstuk 3). Net als geobserveerd door Hobbs *et al.* was het aantal prolifererende cellen en bloedvaten binnen de tumor significant toegenomen als asTF tot overexpressie kwam. Deze toegenomen tumorexpansie door asTF expresssie werd zeer versterkt wanneer pellets die estrogeen vrijgeven onderhuids in de muis werden geplaatst (hoofdstuk 4), wat wederom duidt om een synergie van de signaleringsroutes van asTF en de estrogeen receptor in borstkankercellen.

Aangezien asTF en flTF vaak samen tot expressie komen in tumorcellen wilden we vaststellen wat de unieke rol is van asTF gedreven tumorprogressie in de aanwezigheid van flTF. De MDA-MB-231mfp cellijn is verkregen uit MDA-MB-231 cellen die als xenograft in de borstklier van een SCID muis zijn geplaatst, wat leidde tot een agressievere borstkankercellijn. MDA-MB-231mfp cellen bleken afdoende hoeveelheden van beide TF isovormen tot expressie te brengen. Verder verminderde β1 integrine-remmende antilichamen en asTF-specifieke antilichamen de cel proliferatie. Ook werd *in vivo* tumor expansie geremd door asTF blokkade net als het aantal prolifererende cellen, terwijl de dichtheid van micro-bloedvaten onveranderd bleef.

Expressie van asTF in kanker en het klinische beeld

In hoofdstuk 3 presenteerden wij de eerste grote studie die wijdverbreide asTF niveaus in borstkanker monsters liet zien: asTF was aanwegig in >95% van de tumor monsters (n=574), terwijl gezond borstweefsel maar in 4% van de gevallen positief aankleurde. Hoge asTF expressie was geassocieerd met een hogere histologische graad en tumor grootte. AsTF was niet geassocieerd met oestrogeen receptor (ER) status, maar in hoofdstuk 4 zagen we in borstkanker duidelijk overlap tussen asTF afhankelijke genexpressie profielen en ER-afhankelijke genregulatie. Toen we de associaties tussen asTF expressie en klinische parameters her-evalueerden door de tumor monsters te stratificeren voor ER-status, observeerden we een associatie tussen asTF expressie en tumor graad in ER+ maar niet in ER- tumoren. Aangezien flTF expressie geassocieerd was met tumor graad in ER+ en ER- tumoren, suggereerde deze observatie dat asTF, en niet flTf, samenwerkt met de ER-signaleringsroute in borstkanker.
In hoofdstuk 7 rapporteerden wij wijdverbreide asTF expressie in een groot cohort colorectale tumor monsters (n=353). Al werd er geen associatie met

tumor graad gevonden, hoge asTF expressie bleek wel geassocieerd met een bijna-significante vermindering van ziektevrije overleving (p=0.050). AsTF expressie was geassocieerd met non-mucineuze tumoren, was weer geassocieerd is met toegenomen terugkeer van de tumor en verminderde overleving.

Tumor-geproduceerd Factor VII versterkt tumor progressie via het flTF:FVIIa:PAR2 complex

De rol van flTF in bloedstolling en oncogene processen is tot in detail uitgezocht: de formatie van het binaire flTF:FVIIa complex op een fosfolipide oppervlak leidt tot PAR2 activatie. De expressie van flTF op de oppervlakte van tumorcellen is tot in detail bestudeerd, maar hierbij wordt aangenomen dat het FVII afkomstig is uit plasma. Al is FVII uitbundig in plasma aanwezig, de vroege ontwikkeling van een tumor word juist gekarakteriseerd door gebrekkige blootstelling aan de circulatie, waardoor een hypoxisch milieu ontstaat. In 2006 raporteerden Koizume *et al.* dat tumorcellijnen FVII ectopisch synthetiseerden, en dat dit FVII cel migratie reguleerde in samenspraak met flTF. Verder bleek de ectopische expressie van FVII te worden versterkt onder hypoxische omstandigheden, wat een rol voor ectopisch FVII suggereert in tumor progressie mogelijk voordat angiogenese plaatsvindt. Een ander mechanisme voor geïnduceerde FVII expressie is voorgesteld door Naderi *et al.,* zij rapporteerden de co-expressie van de androgeen receptor (AR) en FVII in een aantal borstkankercellijnen, en lieten zien dat AR activatie ectopische FVII synthese induceert en de pro-coagulante activiteit verhoogd.

Bewijs voor de expressie van ectopisch FVII in tumor monsters is tot nu toe sporadisch gerapporteerd. In een studie naar twintig baarmoederhalskanker monsters kwam FVII samen met TF tot expressie. In een colorectaal kanker cohort (n=55) werd FVII expressie op eiwit- en mRNA niveau gerapporteerd, en bleek dit met lever uitzaaiingen geassocieerd te zijn. In hoofdstuk 5 rapporteren we dat uit een groot cohort (n=574) borstkanker patiënten 39% van de tumor monsters positief aankleurden voor FVII. Om uit te sluiten dan een positieve FVII kleuring werd veroorzaakt door FVII dat door de tumor was opgenomen uit de circulatie hebben we de FVII mRNA expressie onderzoek in een subgroep van de monsters. FVII-eiwit expressie bleek geassocieerd te zijn met het FVII mRNA niveau, tumor graad, ER en PgR status, TGFβRII, TGF receptor

1 en 2, en flTF. Verder bleek FVII expressie geassocieerd met verminderde overleving van de patiënten. Dit effect op overleving was nog sterker als ook flTF tot expressie kwam, wat suggereert dat het flTF:FVII complex dat door de cel zelf gesynthetiseerd wordt al kan leidden tot ziekte progressie, mogelijk voordat de tumor voldoende is blootgesteld aan plasm waar FVII (gesynthetiseerd in de lever) in ruime mate aanwezig is.

Al vonden we in hoofdstuk 5 in borstkanker een duidelijke associatie tussen FVII expressie en progressie, deze verhouding bleek niet geheel op te gaan in colorectaal kanker: in hoofdstuk 7 observeerden we hoge FVII expressie in 80.1% (n=237) van de colorectale kanker monsters, maar FVII expressie bleek niet geassocieerd te zijn met de overleving van patiënten of met het tumor stadium. FVII expressie werd echter ook gedetecteerd in 53 van de 60 (88.3%) monsters die geclassificeerd waren als laag in FVII expressie, wat mogelijk verklaard waarom er geen significant effect tussen FVII expressie en het klinische beeld gevonden werd. Hoge FVII expressie was wel geassocieerd met non-mucineuze tumoren en micro-satelliet instabiliteit, een combinatie die in een meta-analyse uit 2012 weer geassocieerd werd met verminderde overleving.
Al hebben tumoren van verschillende origine verschillende onderliggende moleculaire processen die oncogenese aansturen, is het toch verleidelijk om een algemene hypothese op te stellen over hoe de expressie van ectopisch FVII door tumorcellen de tumor progressie kan aanjagen. Koizume *et al.* lieten zien dat FVII *in vitro* wordt gesynthetiseerd onder hypoxische omstandigheden. Aangezien de vroege stadia van tumor ontwikkeling zich kenmerken door een gebrek aan vascularisatie en met als gevolg een gebrek aan (uit de circulatie afkomstig) FVII, kan ectopische FVII expressie de signalering van het flTF:FVIIa complex mogelijk maken voordat afdoende blootstelling aan de circulatie plaatsvind. Hierdoor kan de tumor een voorsprong krijgen op het gebied van angiogenese en metastasering, wat zich weer kan vertalen tot verminderde overleving in patiënten. Aangezien eigenlijk alle tumor monsters uit patiënten al een vasculair netwerk hebben en dus zijn blootgesteld aan FVII uit de circulatie, is dit niet het ideale materiaal om ectopisch FVII-gedreven tumor biologie te bestuderen. Ook wordt de grootte van tumoren in xenograft modellen met een schuifmaat door de huid heen gemeten, waardoor de detectie en het correct opmeten van kleine tumoren minder precies is. Hierdoor is deze techniek niet uitermate geschikt om tumorgroei te meten in tumoren waar angiogenese

nog niet heeft geleid tot een ontwikkeld vasculair netwerk geschikt voor exponentiele tumorgroei. In hoofdstuk 5 observeerden we dat tumorgroei van MDA-MB-231 cellen (die flTF tot expressie brengen) enigszins geremd werd als er FVII expressie in de lever geblokkeerd was. Al was dit effect minimaal, dit versterkt wel de hypothese dat circulatie- en tumor-FVII beide in staat zijn tumorgroei te versterken, al kan ectopische FVII expressie in een vroeg stadium tot een lokaal hogere concentratie leiden als de blootstelling aan de circulatie nog beperkt is.

Conclusies en toekomstige perspectieven

Dit proefschrift bevat een overduidelijke hoeveelheid bewijs dat flTF expressie is geassocieerd met verminderde overleving in verscheidende typen solide tumoren. Ectopische expressie van FVII in borsttumoren leidt ook tot significant verminder overleving. *In vitro* en *in vivo* experimenten laten beide zien dat ectopische FVII expressie de signalering van flTF bevordert en resulteert in toegenomen tumorcelinvasie, tumorgroei en metastasering, onafhankelijk van FVII uit de circulatie. Samenvattend versterkt ectopisch FVII de effecten van flTF signalering in kanker progressie, mogelijk voordat angiogenese heeft geleid tot een ontwikkelde vasculaire verbinding met de bloedsomloop. Een andere mogelijke hypothese is dat tumorcellen die flTF, FVII en PAR2 tot expressie brengen functionele intracellulaire complexen kunnen vormen waardoor de signalering sterker is dan membraangebonden flTF:FVIIa signalering via PAR2. Dit kan de observatie uit hoofdstuk 7 verklaren dat MDA-MB-231 cellen die FVII tot expressie brengen hoge mRNA niveaus van pro-angiogene cytokines produceren dan MDA-MB-231 cellen blootgesteld aan een hoge dosis recombinant FVIIa. Maar gezien dat membraangebonden flTF:FVIIa complexen worden geïnternaliseerd door cellen is het moeilijk om onderscheid te maken tussen flTF:FVIIa complexen die intracellulair gevormd zijn of complexen die geïnternaliseerd zijn, waardoor deze hypothese lastig is te bewijzen.

FlTF en FVII co-expressie in borstkanker was geassocieerd met verminderde overleving van patiënten, waardoor flTF en FVII beide een interessante optie zijn voor therapeutische interventie. In een klinische studie waar een TF-antilichaam werd onderzocht werden geen ernstige bloedingen gerapporteerd, al werden milde bloedingen wel in de dosis-afhankelijk mate beschreven. Het monoclonale flTF antilichaam 10H10 zou daardoor een een interessante optie zijn als klinische interventie om flTF

signalering te remmen, aangezien 10H10 zeer effectief flTF en β1 integrine interactie kan remmen zonder dat de bloedstolling geremd wordt.

Of asTF dat in tumoren tot expressie komt de overleving van patiënten beïnvloed blijft tot op heden onduidelijk. In borstkanker is geen relatie tussen asTF en overleving geobserveerd, al was hoge asTF expressie in colorectaal kanker duidelijk geassocieerd met een verminderde overleving in de eerste 200 maanden. AsTF blijkt een potente drijfveer van tumorcel proliferatie via een unieke subset van integrines, terwijl flTF geen rol lijkt te spelen in het versterken van cel proliferatie. Dit proefschrift biedt nieuwe inzichten in de respectievelijke bijdrage van de TF isovormen flTF en asTF, maar tot op heden is het nog onduidelijk of er een mogelijke (functionele) interactie is tussen flTF en asTF waardoor tumorprogressie wordt gestimuleerd. De co-expressie van flTF en asTF is vaak geobserveerd in tumor monsters, wat dit een interessant aangrijpingspunt maakt voor toekomstig onderzoek.

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Curriculum Vitae

De auteur van dit proefschrift is geboren op 8 juni 1985 te Leiden. Van 1997 tot 2003 heeft hij met goed gevolg zijn vwo-diploma (profiel Natuur & Gezondheid) behaald aan het Rijnlands Lyceum Oegstgeest. In 2003 is hij begonnen aan de bachelor opleiding Psychologie aan de Universiteit Leiden waar hij in 2004 een propedeusediploma behaalde. In 2004 vervolgde hij zijn academische opleiding in het LUMC met de studie Biomedische Wetenschappen. Zijn bachelorstage werd gelopen bij het Einthoven laboratory for experimental vascular medicine onder begeleiding van prof. dr. Pieter H. Reitsma, waar hij genetisch onderzoek heeft gedaan naar hoge proteïne C niveaus in plasma.

Tijdens zijn eerste masterstage op de afdeling Pathologie in het LUMC heeft de auteur onderzoek gedaan naar het mechanisme achter het verlies van p16 expressie in osteosarcoma, onder begeleiding van dr. Anne-Marie Cleton-Jansen. De tweede masterstage vond plaats onder leiding van dr. Robert Passier van de afdeling Anatomie & Embryologie in het LUMC, waar een duaal fluorescent reporter stamcel model werd ontworpen om de ontwikkeling van stamcel naar cardiomyocyt te bestuderen.

In december 2010 begon de auteur aan een promotieonderzoek op het Einthoven laboratory for experimental vascular medicine onder begeleiding van prof. dr. Henri H. Versteeg en prof. dr. Pieter H. Reitsma. Dit onderzoek richtte zich op het ontrafelen van de respectievelijke bijdrage van Tissue Factor isovormen en Factor VII op meerdere facetten van tumor biologie.

Van juli 2015 tot februari 2017 heeft hij gewerkt aan internationale klinische studies naar o.a. bloedingsziekten als Clinical Research Associate, bij Quintiles en vervolgens Chiltern. Sinds maart 2017 is de auteur als Manager Business Development werkzaam bij Medicapital Rent. Hij woont in Leiden met zijn vriendin Ana.