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Coagulation factor V and doxorubicin in breast cancer

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Sammendrag

Sammenhengen mellom kreft og trombose er velkjent, likeså er sammenhengen mellom koagulasjonsaktivitet og tumorprogresjon. Ved å få økt kunnskap om de underliggende molekylære mekanismene i disse prosessene, vil man kunne oppnå en bedre tilpasset behandling for de pasientene som lider av kreft eller av kreftrelatert trombose. De prokoagulante og de antikoagulante egenskapene til koagulasjonsfaktor V (FV) er velkjente, men sammenhengen til kreft er ennå ikke studert.

Målet for denne masteroppgaven var å oppnå en bedre forståelse av de underliggende mekanismene som knytter FV og kreft sammen. I første omgang konstruerte vi en FV overekspresjonsmodell for å kunne studere effekten av FV i brystkreftceller. I tillegg studerte vi virkningen av FV i brystkreftcellene MDA-MB-231, MCF-7 og SUM102. Vi ønsket å studere effekten doxorubicinbehandling og hemming av p53 hadde på produksjonen av FV i cellene, samt effekten av FV i forhold til celle-overlevelse.

FV overekspresjonsmodellen viste seg dessverre ikke å være optimal og viste et høyt utrykk av *F5* mRNA, men med lave nivåer av FV proteiner. FV overekspresjonsmodellen inneholdt to nye mutasjoner. Den første, i ekson 2, som endret et spleisesete og den andre, en synonym mutasjon i ekson 13. Ved å rette opp mutasjonen i ekson 2 viste det seg at denne hadde en effekt på det uttrykte mRNA nivået av *F5*, men mutasjonen påvirket tilsynelatende ikke proteinnivået. Det var ikke mulig å rette opp den andre mutasjonen og derav ble det heller ikke mulig å studere om denne har en innvirkning på utrykket av FV mRNA eller protein. Ved å bruke FV overekspresjonsmodellen oppnådde vi ikke høye nok FV verdier på proteinnivå til å kunne bruke denne modellen videre i *in vitro* studier

Med hensyn til *F5* mRNA uttrykk i brystkreftceller som ble eksponert for doxorubicin, viste cellene en økning i mRNA uttrykk sammenliknet med ubehandlede celler. Denne økningen i uttrykt *F5* mRNA ble videre påvirket av hemmingen av p53 som førte til en senkning av *F5* mRNA uttrykket i brystkreftcellene. I tillegg førte en hemming av FV, før cellene ble behandlet med doxorubicin, til økt levedyktighet sammenliknet med cellene som var eksponert for doxorubicin, der FV ikke ble hemmet. Resultatene indikerer at FV har en sammenheng med doxorubicin indusert celledød og har en sammenheng med p53 aktivitet. Disse resultatene gir oss en økt kunnskap og dypere forståelse av hvilken rolle FV spiller i brystkreftceller.

Abstract

There is a well-known link between cancer and the risk of thrombosis. Moreover, the association between increased coagulation and tumour progression is also well established. In gaining a better understanding of the underlying molecular mechanisms, one can obtain a more individualized treatment for patients suffering from cancer or cancer-related thrombosis. In this regard, the coagulation factor V (FV) has not yet been studied, although its procoagulant and anticoagulant properties is well known.

Throughout this thesis, we therefore aim to gain a better understanding of these underlying mechanisms relating coagulation FV and cancer. A FV overexpression construct was made to study the effect of FV in breast cancer cells. The breast cancer cells were also tested concerning an effect of doxorubicin treatment and p53 inhibition upon FV expression, as well as cell viability upon FV inhibition.

The study performed for this thesis, revealed an FV overexpression vector with a high *F5* mRNA expression, but low levels of secreted FV protein levels were measured. The FV overexpression construct contained two novel mutations, where the first, in exon 2, was changing a splicing site and the second, in exon 13, was a synonymous mutation. Correction of the first mutation in exon 2 showed to influence the *F5* mRNA expression but not the expressed FV protein level. The second mutation was not possible to correct and its effect was therefore not possible to study. The construct was unsuccessful in gaining a high enough FV expression at protein levels for further studying the functional effects of FV in cancer. Results regarding *F5* mRNA expression when breast cancer cells were exposed to doxorubicin, revealed an increase in mRNA expression compared to non-treated cells. This increase in *F5* mRNA expression was further affected by the inhibition of p53 which was leading to a decrease in *F5* mRNA expression in the breast cancer cells. Further the inhibition of FV in the same breast cancer cells, before treated with doxorubicin, led to an increase in cell viability compared to non-inhibited cells exposed to doxorubicin. These results indicate that FV plays a role in the doxorubicin induced cell death, associated with the p53 pathway, and give us better knowledge and understanding of the role of FV in breast cancer cells.

Abbreviation

A	Adenine
ABCB1	ATP Binding Cassette Subfamily B Member 1
ABCC1	ATP Binding Cassette Subfamily C Member 1
APC	Activated protein C
ARG	Arginine
AT	Antithrombin
Bp	Base pair
cDNA	Complementary DNA
CT	Threshold cycle
ddNTP	Dideoxyribonucleotide
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
E.coli	*Escherichia coli*
EC	Endothelial cells
ELISA	Enzyme Linked immunosorbent assay
EPCR	Endothelial protein C receptor
ER	Estrogen receptor
F	Factor
F5	Factor V gene
FV	Factor V protein
FVa	Activated factor V
FVac	Activated anticoagulant factor V
G	Guanine

HER2	Human epidermal growth factor receptor 2
IL	Interleukin
mRNA	Messenger ribonucleic acid
NTC	No template control
p53	Tumour protein 53
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFT-α	Pifithrin α
PR	Progesterone receptor
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
qRT-PCR	Real time quantitative PCR
SD	Standard deviation
SEM	Standard error of the mean
T	Thymine
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
Top2	Topoisomerase II
Wt	Wild type

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1. Introduction

1.1 Haemostasis

1.1.1 Haemostasis under normal conditions

Haemostasis is the balanced process in the body that prevent bleeding while keeping the blood fluid and circulating in the system. A strict regulation of the haemostatic system is essential to maintain the blood in a fluid state. This balanced regulation between procoagulants and anticoagulant forces prevents haemorrhage (bleeding) and blood clot formation (Mackman et al. 2007). There are multiple contributing cofactors, and the process has been described in several ways. One well established description is the cell based model of coagulation (Smith 2009; Versteeg et al. 2013).

1.1.2 Cell based model of coagulation

The cell based model of coagulation is an activation of a cascade of several coagulation components (Figure 1). This cascade, including its cellular components, was originally developed when the waterfall/cascade theory failed to give a good explanation regarding the coagulation process *in vivo*. Activation of the cascade is initiated by the rupture of the endothelial cell (EC) layer in a blood vessel. This rupture exposes the subendothelial matrix to blood, revealing several platelet binding proteins like tissue factor (TF) and initiating the formation of clots covered by a fibrin mesh (Smith 2009; Versteeg et al. 2013)

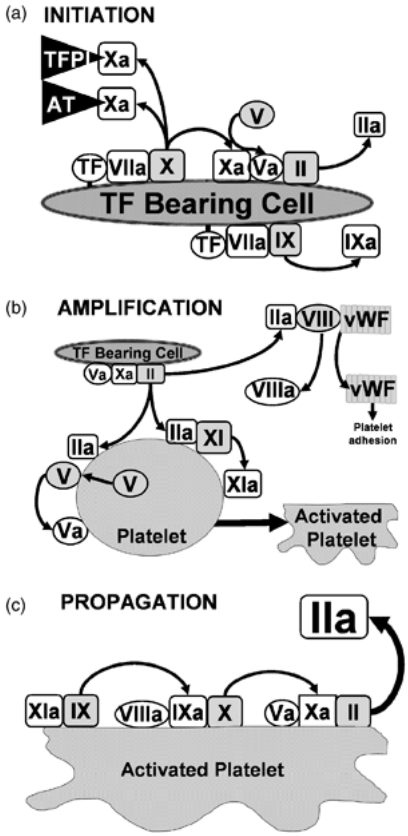

Figure 1. Cell based model of coagulation. a) *Initiation:* TF is exposed to blood upon an injury in the endothelium. This exposure leads to generation of some FXa and thrombin. b) *Amplification:* In this stage FV, FVIII, FXI and platelets are activated by thrombin. c) *Propagation*: Multiple coagulation complexes gather, tenase complex (FIXa-FVIIIa) activates FX to FXa, which combines with FV creating the prothrombinase complex. The prothrombinase complex converts prothrombin to thrombin, the burst of thrombin is essential for the generation of fibrinogen to fibrin. Fibrin binds platelets and blood cells to create a clot preventing the bleeding (Smith 2009).

The cell based model of coagulations (Figure 1) explained in short is initiated by the exposure of TF bound to the subendothelial cells. TF is revealed when an injury in the endothelium cell layer occurs. When exposed, the coagulation factor VII (FVII) binds to the membrane-bound TF which further leads to the activation of FVII to activated factor VII (FVIIa). The cascade continues by the further activation of FXa and FIXa by their cofactor FVIIa. When FXa is activated, it combines with activated factor V (FVa) creating the prothrombinase complex that cleaves prothrombin to thrombin. Several cofactors then bind and are activated by the cascade, which results in the transformation of fibrinogen to fibrin. The fibrin mesh bind to the activated platelet and form a clot, preventing bleeding at the injured sight (McMichael 2012; Smith 2009; Versteeg et al. 2013). After clot formation, a strictly regulated clot dissolution is essential to maintain haemostatic balance. This regulation is controlled by several activators, cofactors and inhibitors (Chapin & Hajjar 2015).

1.1.3 Regulation of coagulation

Avoidance of excessive and noxious coagulation is essential and is controlled by anticoagulant pathways. There are several important inhibitors of this process: antithrombin (AT), tissue factor pathway inhibitor (TFPI), and protein C are key inhibitors with different influences on the anticoagulant pathway (Smith 2009) (Figure 2).

AT is a serine protease inhibitor which is important in keeping the concentration of thrombin and FXa local and not circulating systemically in the blood (Fuchs et al. 1982). It has the ability to inhibit or neutralize multiple activated coagulation enzymes including FIXa, FXa, TF-FVIIa and thrombin (Chuang et al. 2001; Versteeg et al. 2013) .

TFPI is a natural anticoagulant regulating the generation of thrombin by binding to free FXa or the TF-FVIIa complex, resulting in decrease in thrombin (Hackeng et al. 2009). When TFPI binds to Fxa, creating a TFPI-FXa complex, the complex can further bind through

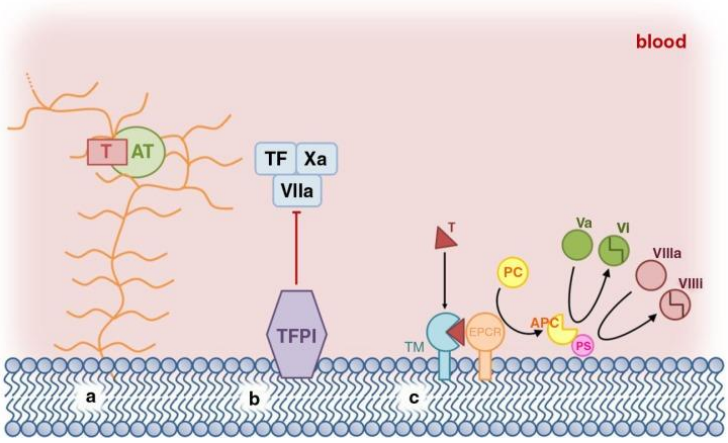

Figure 2. Anticoagulant characteristics. a) cell bound proteoglycans serves as an enhancer of the inhibitor AT. b) TFPI inhibits the TF from initiate coagulation and binds to VIIa-TF, a further binding of FXa strengthen the inhibition and neutralization by TFPI. c) protein C anticoagulant pathway is a cascade inhibiting thrombin by thrombodulin, which enabling the activation of PC through EPCR. The release of aPC from EPCR enables binding of aPC and protein S and inactivate FVa and VIIIa (Bouvy et al. 2014).

another domain to the TF-VIIa complex. The two complexes together form a quaternary inhibitory complex (TF-FVIIa-FXa-TFPI) (Bajaj et al. 2001; Broze 1995). It has also been shown that protein S facilitates inhibition by binding to TFPI and increasing its inhibitory activity (Hackeng et al. 2009). TFPI is capable of inhibiting FVa in the prothrombinase complex, however, this only occurs when FVa is activated by FXa and in some of the

platelets activated FVa. This is because of the acidic portions of the B-domain is preserved in FVa cleaved by FXa (Maroney & Mast 2015).

Prevention of thrombosis in microcirculation is thought to be mainly facilitated by the protein C anticoagulant pathway (Kisiel 1979). Normal activated protein C (APC) must first detach from the endothelial protein C receptor (EPCR) and bind to its cofactor protein S before its activated as an inhibitor (APC-protein S) (Versteeg et al. 2013; Walker 1980). The FVa and FVIIIa are inactivated by the APC-protein S complex, which in addition serves as an inhibitor for tenase and the prothrombinase complex. Intact FV can serve as a cofactor for APC in the inactivation of FVIIIa and FVa. Cofactor FV is then combined with cofactor protein S, and the combination of these to cofactors facilitates APC in its inactivation process (Versteeg et al. 2013).

1.2 Cancer and coagulation

1.2.1 Hallmarks of cancer

According to Hanahan and Weinberg (2011), there are six biological hallmarks of cancer required for tumour growth (Figure 3 left), in addition to two emerging hallmarks and two enabling characteristics (Figure 3 right). The cell must exhibit the complete set of hallmarks before cancer is fully developed (described below).

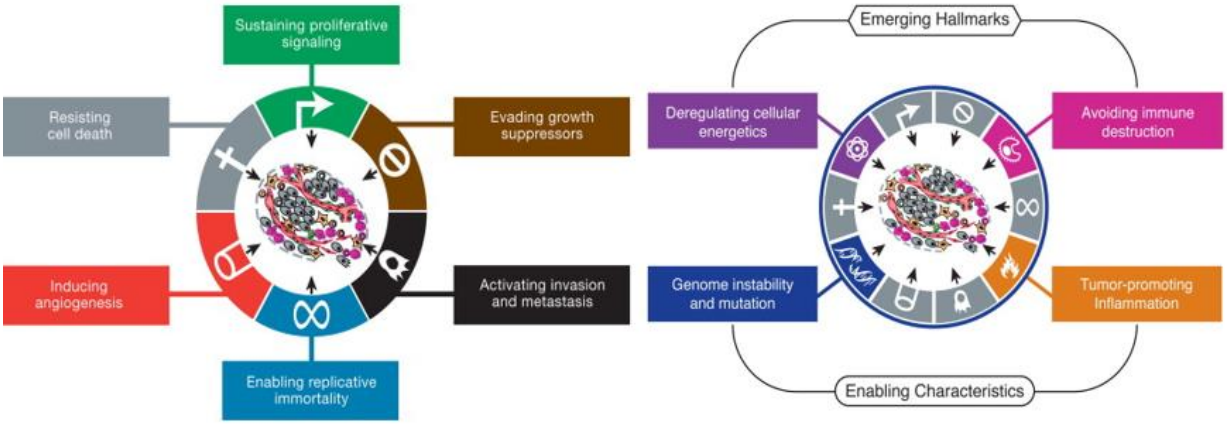

Figure 3. The hallmarks of cancer. Illustration of the six biological hallmarks of cancer (left), in addition to the two emerging hallmarks (right) and two enabling characteristics (right) (Hanahan & Weinberg 2011).

The six biological hallmarks of cancer consist of (Figure 3 right):

1. **Sustaining proliferative signalling:** Cancer cells produce their own growth signals and do not depend on stimulation from external signals, unlike normal cells.
2. **Evading growth suppressors:** Cancer cells are resistant to tumour suppressor genes like tumour suppressor protein 53 (p53), which would otherwise prevent cell growth and division.
3. **Enabling replicative immortality:** Cancer cells are capable of infinite growth and division, by being able to overcome shortening of the telomeres.
4. **Inducing angiogenesis:** Cancer cells induce pro-angiogenic factors, stimulating blood vessel formation and increasing blood flow with oxygen and nutrition supply.
5. **Resisting cell death**: Cancer cells can resist programmed cell death (apoptosis), while normal cells are programmed to undergo apoptosis upon damage.
6. **Activating invasion and metastasis:** Cancer cells are able to spread from their origin to other parts in the body, for example by intravasation of blood or lymphatic vessels.

The two emerging hallmarks consists of (Figure 3 right):

1. **Deregulating cellular energetics:** Cancer cells exhibit the Warburg effect, generating energy from abnormal metabolic pathways.
2. **Avoiding immune destruction:** Cancer cells have the ability to avoid the body's own immune system.

The two enabling characteristics consist of (Figure 3 right):

1. **Genome instability and mutation:** Cancer cells have genetic mutations which evolve during growth and generally lead to chromosomal abnormalities.
2. **Tumour-promoting inflammation**: Cancer cells are induced by inflammation, which leads to angiogenesis and induced blood flow.

1.2.2 Cancer and thrombosis

The relationship between cancer and thrombosis has been known, recognized since the late 19th century (Trousseau 1865). Over the ensuing decades, multiple studies continued to support a link between cancer and an increased risk of venous thrombosis (VT) (Blom et al. 2005; Heit et al. 2000; Laporte et al. 2008; Nand et al. 1987). Tumour cells have the ability to activate the coagulation cascade, since they produce and release procoagulants causing thrombosis (Caine et al. 2002; Falanga & Rickles 1999; Gouin-Thibault et al. 2001).

Cancer associated VT is the second most frequent cause of death in cancer patients (Khorana et al. 2007). The risk of cancer associated VT varies greatly among the different types of cancers. The highest risk is found in pancreas cancer (8%), kidney and ovary cancers (6%), and in lung, stomach and brain cancers (5%) (Khorana et al. 2007). Breast cancer, however, carries one of the lowest risks of associated VT at approximately 2% (Chew et al. 2007; Khorana et al. 2007). Breast cancer cells have shown not only to have the capacity of inducing procoagulants, but reduce anticoagulants; this increases risk of thrombosis (Caine et al. 2003).

The bidirectional link between cancer and thrombosis is further emphasised by the findings of increased risk of cancer in patients with thromboembolism (Otten & Prins 2001; Prandoni et al. 1999). Three separate population studies from Denmark, Scotland and Sweden support this, each reporting an increased risk for cancer in patients diagnosed with thromboembolism (Baron et al. 1998; Murchison et al. 2004; Sørensen et al. 1998). The discovery of platelets or fibrin in tumours by histopathological studies also supports a bidirectional link between cancer and thrombosis. Based on this discovery, the clotting mechanism is thought to influence tumour growth, and the tumour cells are thought to have an effect on local activation of the coagulation process (Costantini & Zacharski 1993).

1.2.3 Coagulation and cancer progression

The process of tumour growth and spreading is supported by tumour-specific prothrombotic properties (Figure 4). These properties can be divided into coagulation dependent properties and coagulation independent properties. Coagulation dependent properties of the tumour progression include the formation of either fibrin or thrombin. Thrombin is the final step in the coagulation cascade, while fibrin formation is the final step in clot formation. Coagulation independent properties, however, can interfere with the malignant tumour process (Rickles & Falanga 2001).

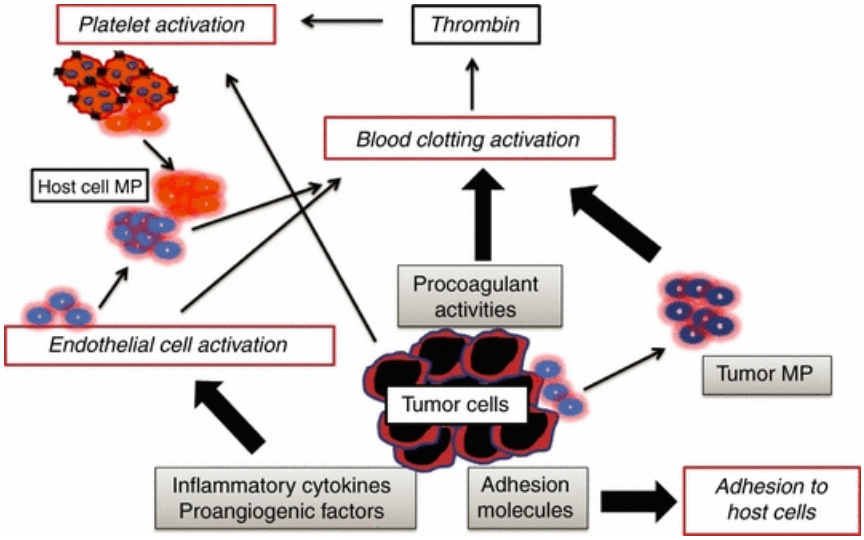

Figure 4. Interactions of tumour and haemostatic system. Tumour cells may activate platelets and endothelial cells, either by direct contact or by release of procoagulant factors. Platelets, together with polymorphonuclear leucocytes, release tumour growth factors and facilitate migration of the tumour cells through the endothelial cell layer. The concomitant activation, together with the ensuing fibrin formation and increased platelet activation, creates a clot (Falanga et al. 2013).

Coagulation dependent pathways and cancer progression

Interactions between platelets and tumour cells play an important role in tumour growth and spreading of cancer cells. Platelets can function as protection for tumour cells against the immune system, by providing a procoagulant surface which promotes localised clotting and coagulation (Bambace & Holmes 2011). This directly activation of platelets also assists tumour cells in migrating trough the vessel wall (Prandoni et al. 2005).

One of the most studied procoagulants expressed by cancer cells is TF (Gale & Gordon 2001). Under normal conditions, TF is the initiator of coagulation when it is exposed hence its expression is being strictly regulated. Malignant cells on the other hand, continuously express TF and initiate coagulation. Cancer procoagulant can activate FX directly without its cofactor VII (Falanga et al. 2013).

Coagulation independent pathway and cancer progression

In the terms of the coagulation independent mechanisms affecting the tumour progression, the non-coagulant effects of TF play an important role (Rickles & Falanga 2001). Tissue factor can modulate vascular endothelial growth factor expression, which influences tumour neovascularisation. This is a particularly important link between cancer progression and activation of coagulation and thrombosis (Shoji et al. 1998).

In addition to the upregulating of TF in cancer cells, there is also a contribution of TF expressed by vascular, stromal and inflammatory cells (Ruf et al. 2011). Cell migration can be promoted through protease-activated receptor (PAR)-2, activated by TF-FVIIa. The TF-FVIIa-PAR-2 pathway further leads to activation of the p44/42 mitogen-activated protein kinase and cofilin pathways. These pathways facilitate migration and invasion by malignant cells (Åberg & Siegbahn 2013).

1.3 Coagulation factor V (FV)

Factor V (FV) is a non-enzymatic cofactor in the generation of thrombin, as a part of the prothrombinase complex (FXa-FVa). It has also been shown that FV is a part of the anticoagulant pathway, where it takes part in the inactivation of activated FVIII. FV is peculiar with this dual effect of activation and inactivation of coagulation, and can lead to both hemorrhagic (bleeding) and thrombotic phenotypes. FV was first discovered by the Norwegian haematologist Paul Owren during World War II (Duga et al. 2004).

1.3.1 Structure and function of coagulation factor V

The gene encoding FV (*F5*) is structured as 80kb located at chromosome 1, consisting of 25 exons and encoding as many as 2224 amino acids (Figure 5) (Asselta et al. 2006; Cripe et al. 1992). This complex gene has a 6-domain structure (A1-A2-B-A3-C1-C2), where removal of the middle B-domain by thrombin or partly removal of the B-domain by FXa generates an

activation of FV to FVa. A cleavage in A2-domain by APC generates activation of FV to anticoagulant activated factor V (FVac) (Asselta et al. 2006).

Up to 20% of FV is stored in platelet α-granules, while the majority of FV is found in plasma, circulating in the blood (Gould et al. 2004; Thomassen et al. 2003). The single chained FV is mainly synthesized by hepatocytes in the liver, although recent studies claim they are also expressed by monocytes (Dashty et al. 2012; Segers et al. 2007). The platelet part of FV is partly proteolyzed and stored in connection to the binding protein multimerin. It is also partially synthesized in megakaryocytes and partially absorbed from plasma through endocytosis (Asselta et al. 2006; Segers et al. 2007).

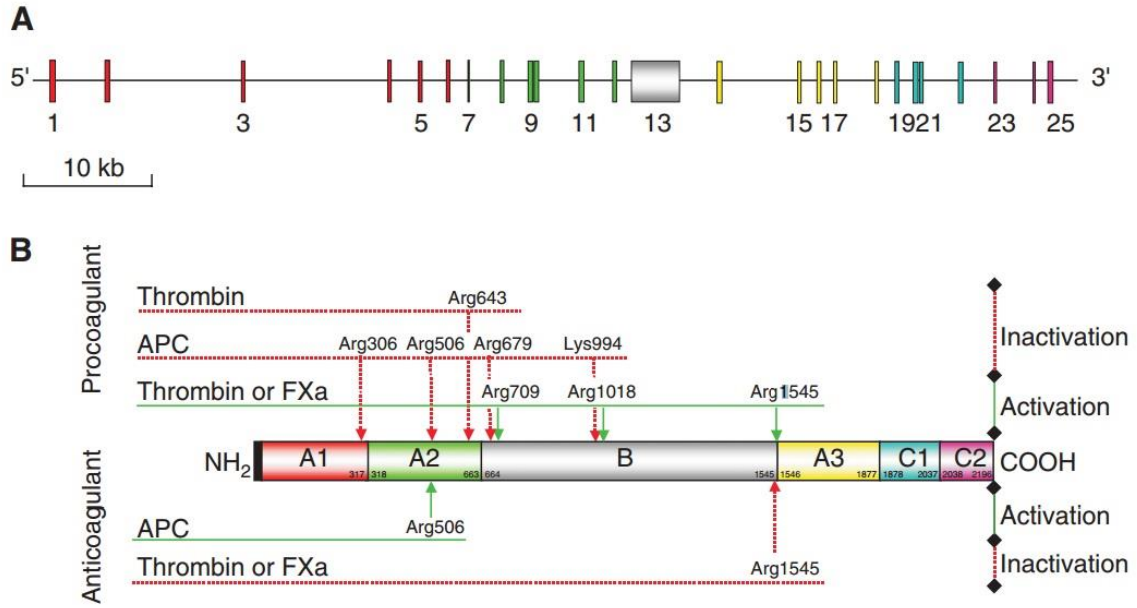

Figure 5. Schematic structure of the FV gene. A) Exons and introns of the FV gene, exons are shown with coloured structures, while introns are shown as a black line. B) FV domains organized by the correlated colored exons (A). FV cleavage sites for activation or inactivation of anti- and procoagulants, are marked by arrows, red represent inactivation and green represent activation. Amino acid numbers are shown for each cleavage site in the FV protein and signal peptide is represented by NH_2 (Asselta et al.2006).

1.3.2 Procoagulant and anticoagulant properties of FV

Whether FV turns into an activated anticoagulant or a procoagulant depends on the concentration of each kind of protease, procoagulant and anticoagulant. For instance, FV will be directed to a procoagulant by the presence of thrombin or FXa, while cleavage by APC turns FV into FVac. FVac may also be converted from an anticoagulant to a procoagulant FVa by FXa or thrombin (Cramer et al. 2010).

Activated FV functions as a cofactor in both the anticoagulant and procoagulant pathways, are shown in Figure 6.

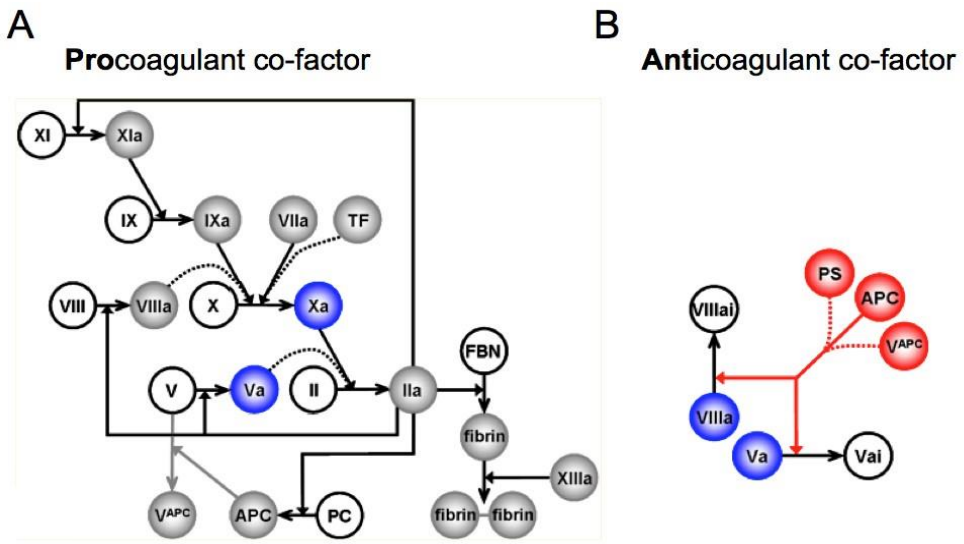

Figure 6. FV activation and inactivation co-factor pathways. A) In the activation of thrombin (IIa) FVa procoagulant function as a cofactor for FXa. B) In the activation of FVIIIa and FVa, FV anticoagulant function as a cofactor for APC (modified from Cramer & Gale 2012).

Procoagulant FV

FV must be activated from FV to FVa to gain procoagulant activity. The increase of local concentrations of procoagulant FV, is initiated when platelet FV is released from the activated platelets upon vascular injury (Segers et al. 2007).

Activation of FV to FVa by thrombin is achieved through proteolysis in several arginine areas in the following order: Arg709, Arg1018 and Arg1545. This leads to the removal of the middle B-domain. FVa consists of the heavy chain (A1-A2) and the light chain (A3-C1-C2) held together by a single calcium ion and hydrophobic interactions (Mann & Kalafatis 2003; Nicolaes & Dahlbäck 2002; Thorelli et al. 1998). The most important cleavage for FV to become a activated procoagulant is the cleavage in Arg1545 (Keller et al. 1995).

In the activation of prothrombin to thrombin, procoagulant FVa serves as a cofactor for FXa in the prothrombinase complex. The amount of FVa activated by thrombin is therefore essential for the generation of the prothrombinase complex. (Keller et al. 1995; Segers et al. 2007; Toso & Camire 2004). The result of this induced activation of thrombin, is an further increase of thrombin activated FVa (Mann & Kalafatis 2003).

Activation of FV by FXa is achieved by the same cleavages as in the activation by thrombin, except for the order in which the cleavage is performed: FXa cleaves the FV in the order Arg1018, Arg709 and Arg1545 (Monkovic & Tracy 1990; Suzuki et al. 1982). The cleavage by FXa leads to a partial loss of the B-domain and the light chain and heavy chain are held together by calcium ions and hydrophobic interactions (Mann & Kalafatis 2003; Nicolaes & Dahlbäck 2002).

Serine protease plasmin is also capable of activating FV to FVa, however, it also possesses a greater ability to inactivate membrane-bound FVa (Lee & Mann 1989).

Procoagulant FVa is down regulated by APC, thrombin or plasmin. The APC inactivate FVa to inactivated FVa (FVai) by proteolysis in Arg506, Arg306 and Arg679 (Figure 4) (Asselta et al. 2006; Duga et al. 2004; Nogami et al. 2014). The first cleavage in Arg506 will only partially inactivate FVa, and merely reduces the co-factor activity to FXa. FV Leiden is a result of cleavage in only Arg506, which can occur if a mutation prevents other cleavages; this cleavage has a larger effect on FVa (Price & Ridker 1997). FV leiden is associated with one of the most common inherited mutations leading to thrombosis. The mutation leads to a resistance in APC, because of the mutation altering the APC cleavage site in the *F5* gene (Rodeghiero & Tosetto 1999). Cleavage in Arg306 is thought to be the most important cleavage for the complete inactivation of procoagulant co-factor FVa (Duga et al. 2004). Protein S is also capable of binding and thus acts as a cofactor to APC, which increase the inactivation of FVa (Duga et al. 2004; Solymoss et al. 1988).

FVa inactivation by thrombin occurs in the presence of ECs. Thrombin then cleaves FVa in Arg643, and weakens the connection between the heavy and light chain of FVa (Asselta et al. 2006). Inactivation by plasmin, however, occurs in a small amount, resulting in an inactivation and loss of activity by cleavage in Arg348, Lys1656 and Arg1765 (Lee & Mann 1989).

Anticoagulant FV

Activation of anticoagulant FV is achieved by cleavage in single-chain FV by APC in Arg506 (Figure 5) (Cramer & Gale 2012; Mann & Kalafatis 2003). As an activated anticoagulant, FVac functions as a co-factor for APC in the degradation of FVIIIa and FVa (Figure 6). To achieve its anticoagulant function, FV depends on the vitamin-K dependent plasma protein, protein S, which also serves as a co-factor to APC together with FVac (Cramer et al. 2010;

Nogami et al. 2014; Thorelli et al. 1999). It is the APC-FVac-protein S complex that inactivates FVa and FVIIIa (Cramer & Gale 2012).

In addition, the C-terminal of the B-domain in FV is essential for the cofactor activity of FVac (Thorelli et al. 1998). The C-terminal of the B-domain is a very acidic region with N-linked glycosylation sites, and it is assumed to be of importance in the binding of FVac to APC and protein S (Cramer & Gale 2012). In contrast to FVa, FVac cannot be activated by FXa, due to the fact that membrane bound single-chain FV does not have the ability to bind FXa and cannot therefore be activated by FXa (Keller et al. 1995; Toso & Camire 2004). FVac is inactivated by the proteolytic cleavage of thrombin or FXa in Arg1545. This cleavage results in loss of the B- and A3-domain in FVac (Figure 5) (Thorelli et al. 1999). The loss of the B-domain, results in the loss of the acidic region with N-linked glycosylation sites, causing FVac to lose its binding affinity in the APC and protein S complex (Cramer & Gale 2012).

1.3.5 Other biological roles of FV

In addition to affect the coagulation cascade, FV is also participates in inflammatory responses. When cleaved by APC in Arg506, it functions together with protein S as a cofactor for APC-mediated anti-inflammatory cell signalling (Liang et al. 2015).

Mutations in FV have also recently been associated with cancer. Homozygote carriers of the mutation FVL showed an increased risk of colorectal cancer (Vossen et al. 2011), and a study by Klee *et al.* (2012) showed an increase of *F5* mRNA levels in prostate tissue. SNPs in the gene FV have been found to have an association with breast cancer and increased risk of thrombosis (Mann & Kalafatis 2003) In addition, studies performed in our research group, revealed an increase in *F5* expression in aggressive breast tumour subtypes and an association with favourable outcomes. The immune modulatory effects were here suggested to be involved in the biological properties of FV in cancer (Tinholt et al. Submitted).

1.4 Breast cancer

1.4.1 Epidemiology and etiology

With over a million devastating cases each year, breast cancer is one of the most common malignancies in the world. It is the second most common cancer globally after lung cancer

(Benson & Jatoi 2012; Ferlay et al. 2013). Although breast cancer incidence is increasing, its mortality rate is decreasing worldwide (Ferlay et al. 2015).

Generally, 5-10% of all incidents are caused by genetics, mainly by *BRCA* genes However, the main risk factor is not genetics, but gender (female) and age (Reeder & Vogel 2008). Multiple other factors have been identified including menopause, lack of or late pregnancy, hormone treatment, obesity and lack of breast feeding (McPherson et al. 2000).

1.4.2 Characterization of breast cancer

There are multiple variations in the molecular and clinical characteristics of breast cancer, making it a heterogeneous disease. Clinical characteristics are divided into subgroups where they are characterized by tumour size, hormone receptor status, lymph node status and histological grade. Estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2) are important in breast cancer classification (Parker et al. 2009). ER and PR are the most common, found in 80% of diagnosed breast tumours. Incidents where both these receptors are present, patients have shown a positive response to hormonal therapy. However, this happens in only about 60% of the ER positive tumours, and the 40% ER positive tumours which are PR negative responds poorly to hormone treatment. 10-15% of the breast cancers diagnosed tend to be triple negative (they lack overexpression for ER, PR and HER2), resulting in a poor prognosis (Vuong et al. 2014).

Breast cancer cells can be subdivided into four intrinsic molecular subtypes: luminal A, luminal B, basal-like and HER2-enriched tumours. These four subtypes can be separated with a distinctive expression profiles (Perou et al. 2000; Sørlie et al. 2001). Histopathological characteristics and molecular tumour subtypes usually correspond. For example, basal-like tumours usually belong to the triple negative subgroup, while luminal-types are mostly ER positive tumours (Vuong et al. 2014). Basal-like and HER2-enriched tumours have the lowest survival and when compared with other subtypes, luminal A tumours have the highest survival rate (Sørlie et al. 2001).

1.5 Cancer treatment

1.5.1 Chemotherapy

Chemotherapy is an overall concept of cancer treatment with drugs. There are several different chemotherapy drugs, affecting different types of cancer. Chemotherapy works by

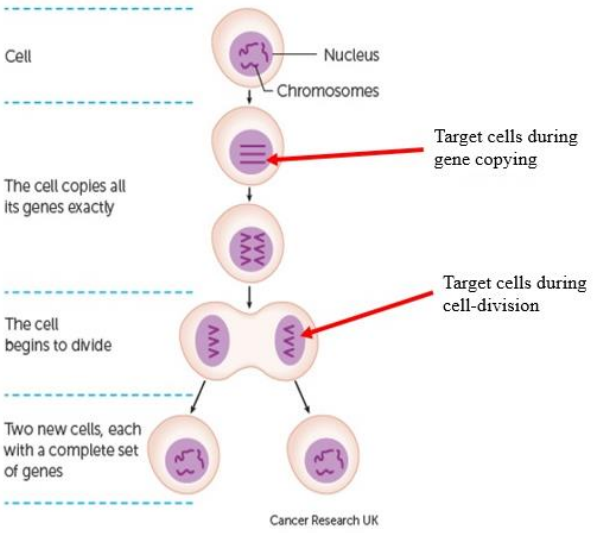

Figure 7. Chemotherapy target in cells. Chemotherapy targets cells under different stages of division, either during gene copying or during cell-division (©CancerResearchUK 2014).

killing cells in the dividing stage (Figure 7). Different drugs target cells in different stages of division, either by targeting the cells' control centre or the chemical processes in the dividing stage (Chabner & Loeffler 2015). Normal resting cells are not likely to be as affected as cancer cells. On the other hand, some cells are continuously dividing in the body (e.g. hair and bone marrow), and these are relatively likely to be affected by chemotherapy. However, cancer cells divide far more rapidly than normal cells, and are therefore more likely to be eradicated by chemotherapy than normal dividing cells in the body (©CancerResearchUK 2014; Corrie 2011).

Cancer cells may be resistant to certain chemotherapy drugs, leading tumour growth to increase or stay steady following treatment. Resistant cells may either express genetic factors that result in resistance to the drug, or my relapse from medical induction or medical selection (Kudoh et al. 2000).

1.5.2 Chemotherapy and coagulation

A known complication of chemotherapy in treating cancers is thromboembolism (Mukherjee et al. 2010). Although cancer is itself associated with increased risk of thrombosis, the risk further increases when patients receive chemotherapy. The magnitude of this increase is dependent on the type of chemotherapy used. Generally, chemotherapy used in breast cancer treatments has been shown to increase the thrombin-antithrombin formation (Swystun et al. 2011). The connection of chemotherapy and induced coagulation has been most studied in breast cancer patients, also revealing that the highest risk seems to be in post-menopausal woman (Letai & Kuter 1999). Doxorubicin is one of the most used chemotherapeutic drugs in breast cancer patients, and a direct relationship between doxorubicin and induced coagulation in cancer patients is well supported (Woodley-Cook et al. 2006). Doxorubicin has also been shown to influence the APC anticoagulant pathway in breast cancer patients (Mukherjee et al. 2010; Woodley-Cook et al. 2006). Chemotherapy-induced thrombosis can be fatal, and knowledge of the mechanisms behind these connections is therefore both medically interesting and potentially life-saving.

1.5.2 Doxorubicin- mode of action and effect on coagulation

Doxorubicin is one of the most common chemotherapeutic drugs used in treating cancer, including breast, bladder, and lung cancers, as well as lymphoma. Doxorubicin induces cell apoptosis (Hilmer et al. 2004) and is dependent on proteins like p53 to trigger cell death (Sun et al. 2016). Binding enzymes like topoisomerase II (Top2) relaxes supercoiled DNA and inhibit repair of cleaved DNA strands (Buchholz et al. 2002; Pommier et al. 2010), inducing apoptosis. Supporting these actions, a study has been performed showing doxorubicin targeting the Top2b in mice (Figure 8) (Zhang et al. 2012). Ashley and Poulton (2009) showed that doxorubicin is capable of binding to mitochondrial DNA. Furthermore, doxorubicin also has the ability to interfere directly with DNA and inhibit transcription (Hilmer et al. 2004). A more recent study by Denard et al. (2012) showed an increase of ceramides in multiple cell lines treated with doxorubicin. Ceramides cleave the CREB3L1, leading to a change in gene expression and affecting levels of proteins such as p21. While, the p21 furthermore, inhibit cell proliferation in cancer cells (Figure 8). The result of adding doxorubicin is that the cell will increase the expression of pro-apoptotic proteins and a decrease in anti-apoptotic proteins (Tacar et al. 2013).

Resistance is becoming a problem in this treatment method, and doxorubicin-mediated signalling pathways are not yet determined (Tacar et al. 2013). Multiple mechanisms are potentially involved in doxorubicin-induced cytotoxicity including DNA interaction, oxidative stress, DNA damage, death receptors activation and altered p53 expression (Dunkern et al. 2003).

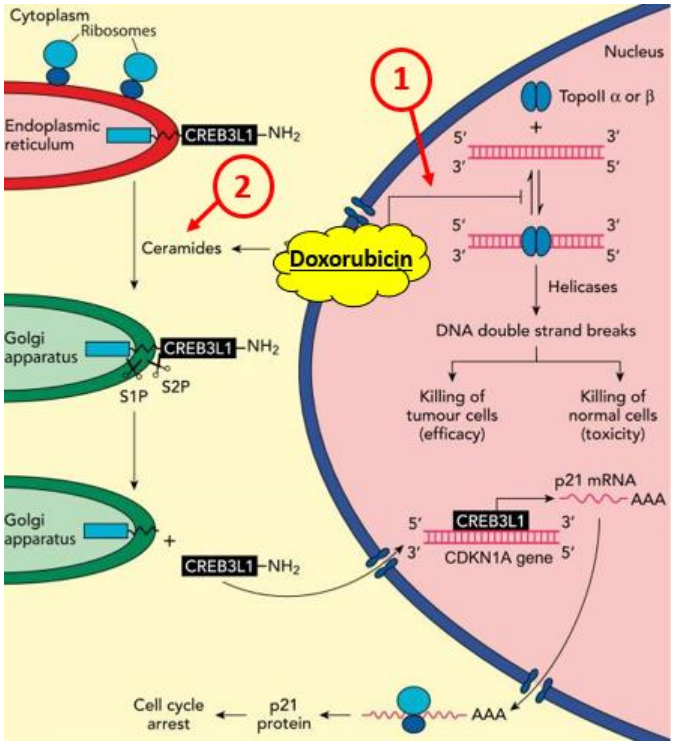

Figure 8. Illustration of two modes of action by doxorubicin. 1) doxorubicin interferes with DNA-transcription, binding topoisomerase II, leading to cutting of the DNA-strands. This action results in cell death. 2) Induced production of ceramides by doxorubicin, a cascade of reactions then follows before the product, an induced expression of p21, inhibiting proliferation in tumour cells. Modified from Patel and Kaufmann (2012).

Multiple transporters are thought to be involved in this resistance mechanism, in particular multidrug resistance protein 1 (ABCB1) and multidrug resistance-associated protein 1 (ABCC1) (Thorn et al. 2011). Inhibitor studies of ABCB1 and ABCC1 have shown a decrease in doxorubicin resistance (Dantzig et al. 2001; Nagata et al. 2002), supporting the possibility that resistance mechanisms involve these transporters. Several studies have also found associations between transporters and resistance to doxorubicin, in different cancer cells (Thorn et al. 2011).

1.5.3 Protein p53 and doxorubicin

If DNA damage occurs, the tumour suppressor gene p53 has an essential role in inducing apoptosis (Wang et al. 2004). p53 is found in various concentrations in all cells and its activation can lead to several outcomes, including apoptosis, cell cycle arrest (transient and sustained) and senescence. Based on these qualities, the protein has been labelled the "guardian of the genome" (Lane 1992). Inducement of p53 will lead to rapid apoptosis or cell cycle arrest, whereas a complete absence of p53 will lead to continuous replication of possibly damaged DNA. p53 is strictly regulated by the cell, and is continuously degraded by mdm2-catalysed proteolysis (Boehme & Blattner 2009).

Lowe et al. (1993) showed that an intact gene of p53 is essential in conducting apoptosis when a cell is exposed to, for example, doxorubicin. Doxorubicin treatment has been shown to induce expression of the p53 gene, contributing to increased cell apoptosis (Shizukuda et al. 2005; Sun et al. 2016). Certain mutations in p53 might result in a resistance in the cells exposed to chemotherapy (Aas et al. 1996): mutant p53 is commonly found in tumour cells, present in as much as 50% of all incidents (Tsang et al. 2005), and it occurs more frequently in ER-negative cells than ER-positive cells (Tsou et al. 2015). These mutant p53 cells seems to express a reduced sensitivity when exposed to chemotherapy (Tsang et al. 2005; Wang et al. 2004), while p53-wildtype (wt) cells shows a trend in high sensitivity to chemotherapy like doxorubicin (Tsou et al. 2015).

2. Aims

The link between cancer and thrombosis is well established, and increased coagulation activity may also support to tumour progression. A better understanding of the association between cancer and coagulation could potentially provide a more individualized therapy against cancer and cancer related thrombosis. In this thesis, the effect of FV overexpression in breast cancer cells, and the effect of doxorubicin in different breast cancer cell lines were studied *in vitro*.

The specific aims of this study were:

I. Study FV overexpression in breast cancer cells.
   a. Construct a new FV overexpression plasmid.
   b. Transfection of FV plasmid
      i. Effect of FV on gene expression and protein levels
   c. Functional effects of FV overexpression
      i. Effect on *IL6* and *IL8* expression
      ii. Effect on p53 activity

II. Study the effect of doxorubicin in different breast cancer cell lines.
   a. Effect on FV expression
   b. Effect on FV through p53
   c. Effect on cell growth

3. Materials and methods

The complete list of solutions, reagents, software, instruments, kits and primers used during this thesis are listed in Appendix 5 and 6.

3.1 Construction of a FV expression plasmid

In order to produce a *F5* cDNA compatible with the restriction sites in vector pcDNA5/FRT, a long-range PCR with primers containing Hind III and Not I overhangs were performed. We possessed a vector containing the entire *F5* gene, plasmid pMT2-FV-wt. This was used as template in the long-range PCR to obtain a complete and a functional *F5* cDNA fragment. The amplified *F5* cDNA and vector pcDNA5/FRT were digested with restriction enzymes *Hind III* and *Not I*, by the use of Thermo Scientific's FastDigest Kit, according to manufacturer's protocol (Table 1). To control the function of the digestion enzymes, three controlled samples were additionally added: undigested vector, digested with only *Hind III* and digested with only *Not I* (Table 2). For digestion, the reactions were incubated at 37°C for 20 minutes to activate the restriction enzymes. For inactivation of the restriction enzymes the reactions were incubated at 80° for 10 minutes. Digested *F5* cDNA was ligated with the opened vector pcDNA5/FRT by Thermo Scientific's Rapid Ligation Kit, according to manufacturer's protocol (Table 3) (Figure 9). To verify the success of the ligation of *F5* cDNA and pcDNA5/FRT control agarose gel was performed[1].

Table 1. Reagents and amounts used in one reaction of digestion of the *F5* cDNA from long-range PCR.

Reagent	**Sample x1 reaction**
F5 cDNA	5 µL
10x Fast Digest Buffer	2 µL
Enzyme	
Hind III	1 µL
Not I	1 µL
Nuclease free H_2O	21 µL
Loading dye	-
Total volume	30 µL

[1] Described in section 3.3.1

Table 2. Reagents and volumes used in one reaction of the digestion of vector pcDNA5/FRT.

Reagent	**Digested with *Hind III* and *Not I***	**Control undigested**	**Control digested with *Hind III***	**Control digested with *Not I***
Vector pcDNA5/FRT	4 µL	4 µL	4 µL	4 µL
10x Fast Digest Buffer	2 µL	-	2 µL	2 µL
Enzyme				
Hind III	2 µL	-	2 µL	-
Not I	2 µL	-	-	2 µL
Nuclease free H_2O	10 µL	15 µL	12 µL	12 µL
Loading dye	-	1 µL	-	-
Total volume	20 µL	20 µL	20 µL	20 µL

Table 3. Reagents and amounts used in one reaction in ligation

Reagent	**Sample x1**
F5 cDNA digested	1 µL
Opened vector pcDNA5/FRT (100ng/µL)	1,8 µL
5xT4	2 µL
T4 enzyme	1 µL
Nuclease free H_2O	4,2 µL
Total volume	10 µL

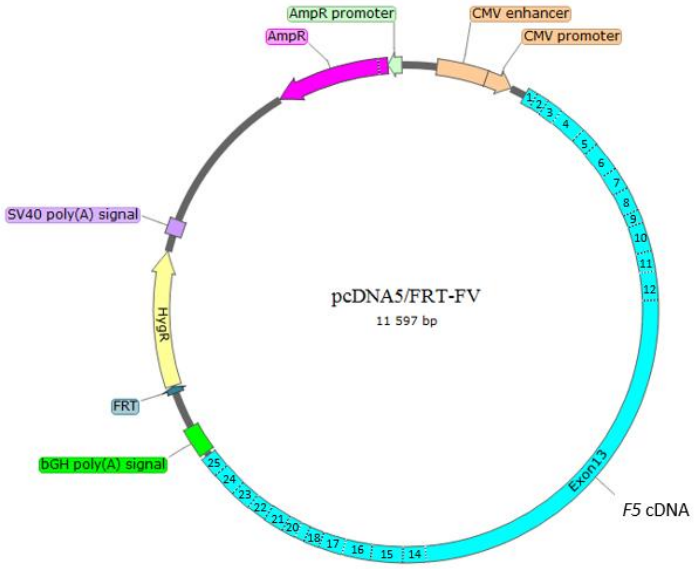

Figure 9. Schematic illustration of the pcDNA5/FRT-FV expression plasmid. The *F5* cDNA with exons displayed in blue and the insert location in the pcDNA5/FRT vector. The size of the vector is 570bp, while the larger inserted *F5* fragment is approximately 80kb. Important areas for cloning and transformation in vector pcDNA5/FRT are shown with highlighted arrows.

3.2 Microbiological techniques

3.2.1 Cloning and transformation

Within molecular biology, transformation is the process of which a cell absorbs and incorporates DNA from its surroundings. This process requires the cell to be in a competent state where it has the ability to absorb DNA. In this thesis One Shot® TOP10 Chemically Competent *Escherichia coli* (*E.coli*) cells were used. The *E.coli* bacterial cells are chemically competent when they have been treated with a calcium chloride ($CaCl_2$) solution, making them permeable for the uptake of DNA. The chemically competent cells are incubated on ice to facilitate the plasmid uptake, and a heat shock from this state opening the cells pores will further facilitate plasmid uptake by the competent cell.

Cloning and transformation in this thesis was carried out according to the manufacturer's protocol from the pcDNA3,1/V5-His TOPO TA Expression Kit. Competent *E.coli* cells were transformed with, the new constructed FV plasmids, pMT2-FV-wt as a positive control plasmid and an empty pcDNA5/FRT vector. 1 µL of each plasmid sample was added to the competent cells and incubated on ice before heat shocking at 42°C, for uptake of plasmids into the cell. The cell solution had 1 hour pre-incubation in SOC-medium before the suspension was spread on LB agar plates[2] (containing 100µg/mL ampicillin), to select for transformed colonies.

Before isolation and purification, amplification of the plasmids was achieved by picking single colonies of transformed cells for each plasmid. The picked colonies were cultured separately in selective Lurian Broth(LB)-medium[3] (containing 100µg/mL ampicillin), and incubated over night at 37°C and 200rpm.

3.2.2 Extraction and purification of plasmid DNA

Extraction and purification of plasmid DNA from a bacterial culture, was performed by a technique where cells are lysed under alkaline conditions. Under alkaline conditions the adding of a neutralizing buffer will keep plasmid DNA stabilized in its native form, and keeping it in the supernatant when centrifuged. Chromosomal DNA and proteins will be

[2] Reagents and volumes used in creation of LB-agar plates are shown in Appendix 6 in Table S11.
[3] Reagents and volumes used in LB-medium are shown in Appendix 6 in Table S10.

denatured under these conditions and form a precipitate when centrifuged. The supernatant is added in a spin column containing a DNA binding filter and binds the plasmid from the supernatant. This is followed by several washing steps to purify the DNA before elution in a collection tube.

Kits for extraction and purification of plasmid DNA vary by the volume of the bacterial culture and corresponding plasmid yield.

In this thesis, the isolation and purification were performed using the Zyppy™ Plasmid Miniprep Kit, used for rapid purification of small samples. ZymoPURE™ Plasmid Maxiprep kit from Zymo research was used for larger samples; it is a more extensive technique and gaining higher DNA concentrations and plasmid quality. The principles for the extraction and purification in these kits are basically the same. However, the incubation time for culturing transfected cells by using Zyppy™ Plasmid Miniprep Kit, is 16-24 hours in approximately 4 mL of selective LB medium containing 100µg/mL ampicillin. While for the ZymoPURE™ Plasmid Maxiprep kit, a pre-incubation is required for 8 hours in approximately 4 mL selective LB medium containing 100µg/mL ampicillin. The culture was then added in approximately 150 mL of the same medium, and incubated for another 16-24 hours. The extraction and purification were performed according to the manufacturer's protocol in both kits.

3.3 Molecular techniques

3.3.1 Agarose gel electrophoresis

Gel electrophoresis is a technique used to separate DNA fragments by size or length. To separate the fragments an electric charge is applied, and the fragments are set to move through the pores of a matrix of chosen substrate, placed in running buffer. In this thesis, a mix of 1% Agarose gel and 10x TAE buffer were used to make the gel, and placed in TAE running buffer. The percentage used in making the gel differs after the fragments size of interest. A higher percentage gives smaller pores for the fragments to migrate through, a smaller fragment will migrate faster and longer than a larger fragment. In addition, supercoiled (circular DNA) and linear DNA will migrate at different speed, and it is therefore possible to detect whether a vector has been opened. In this thesis gel electrophoresis was used to control cDNA fragment size, and to separate fragments form one sample, enabling selective

extraction. A ladder of known sized fragments is added in the edge of the gel, making it possible to detect the fragments size of a sample.

3.3.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a quick simple method to selectively amplify DNA. Using different DNA-polymerases, specific primers for the DNA template and heat cycles, gives PCR several application possibilities.

Long-range PCR

Long-range PCR is optimized for DNA templates with lengths that cannot be amplified using routine PCR methods. Long-range PCR can amplify 30kb fragments with the use of LongAmp® Taq DNA Polymerase, which contains a blend of Taq and Deep VentR™ DNA Polymerases. This combination results in an optimal and stable amplification of large DNA templates.

Long-range PCR was in this thesis used for amplification of *F5* cDNA from the plasmid pMT2-FV-wt before ligation with vector pcDNA5/FRT (Table 4). Samples at 25 µL was amplified using LongAmp® Taq DNA Polymerase at a chosen optimized cycle temperature at 60°C (Table 5). Primers used in this reaction were specific for each end of the *F5* gene and contained sequences for Hind III and Not I overhangs (Table 6). Samples were loaded on to an agarose gel to verify the success of the amplification, the samples were then purified with Wizard® SV Gel and PCR Clean-Up.

Table 4. Composition of reaction mix for long-range PCR for *F5* amplification. Reagent volumes shown for a single long-range PCR reaction only.

Reagents	Volume per sample
Long-range PCR buffer	2,5 µL
dNTP mix (10uM)	1,25 µL
Primer A (Hind III overhang)	0,5 µL
Primer B (Not I overhang)	0,5 µL
LongAmp® Taq DNA Polymerase	0,2 µL
Nuclease free H_2O	19,5 µL
Template (pMT2-FV-wt)	0,5 µL
Total volume	25 µL

Table 5. Cycling parameters for the long-range PCR amplification.

Temperature	Time	Cycles	Stage
93°C	3 minutes	35	Stage 1
93 °C	15 seconds		Stage 2
60 °C	30 seconds		
68 °C	7 minutes		
68 °C	7 minutes		Stage 3
4 °C	Indefinite		

Table 6. Primers used in long-range PCR to obtain restriction sites overhang. Blue color shows extra base pairs, red color shows restriction sites and black color shows *F5* sequence.

Primer names	Primer sequence 5'- 3'
Primer A (Hind III overhang)	ATTCGTAAGCTTGATCTGCCCAGGT
Primer B (Not I overhang)	GCTTAGCGGCCGCAACATTTAACACAGCGTAAAATACA

PCR with DNA polymerase AmpliTaq Gold

AmpliTaq Gold 360 is a PCR master mix for a broad range of targets. It contains hot start enzyme AmpliTaq Gold® 360 DNA Polymerase and 360 GC Enhancer. Using this PCR enzyme, it is possible to amplify sequences up to 5kb.

In this thesis, AmpliTaq Gold 360 was used to control the insertion of the *F5* cDNA fragment in the created FV plasmid. In short, two primers within the *F5* gene was chosen, and added in two different PCR mixes with the FV plasmid as template[4] (Table 7). An empty vector was used as negative control and the plasmid pMT2-FV-wt was used as positive control. Reactions were performed using a previously optimized PCR program (Table 8). After the PCR amplification, the samples were loaded on to a 1% agarose gel for verification of band appearance.

[4] Presented in section 4.1.2: ligation of *F5* cDNA and pcDNA/FRT vector

Table 7. Reagents and volumes used in one reaction for control PCR for *F5* insert. Two reactions for each plasmid, FV plasmid, pMT2-FV-wt and empty vector (pcDNA5/FRT), one reaction for primer 7 and one reaction for primer 9.

Reagents	Volume per sample	Number of samples
Nuclease free H_2O	10,1 µL	
360 GC-enhancer	2 µL	
10x PCR buffer	2 µL	x3 for primer 7
dNTP mix	1,6 µL	
$MgCl_2$	1,2 µL	x3 for primer 9
Primer mix (forward and reverse)	2 µL	
Taq Gold 360	0,1 µL	
DNA~5ng (plasmid)	1 µL	
Totaltvolum	20 µL	

Table 8. Cycling parameters for the Taq Gold 360 enzyme PCR reaction.

Temperature	Time	Stage	cycles
95°C	10 minutes	Stage 1	
95 °C	30 seconds	Stage 2	25
58 °C	30 seconds		
72 °C	1 minutes		
72 °C	7 minutes	Stage 3	
4 °C	Indefinite		

cDNA synthesis

Conversion of RNA to cDNA is accomplished using reverse transcription. In this thesis, the High-Capacity cDNA transcription Kit was used according to manufacturer's protocol. Reverse transcription of RNA to complimentary cDNA is performed prior to real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

During reverse transcriptase random primers bind to the RNA template strand, and is extended by reverse transcriptase, binding dNTPs. Reverse transcription PCR mix was assembled according to manufacturer's protocol, except, the volume used was 2,5 times larger (Table 9). RNA input in the reactions was adjusted to be of the same concentration. RNA was diluted directly in the wells of a 96-well plate, and PCR reaction mix was added directly into each well. Reactions were then run on the 2720 Thermal Cycler with a previous optimized program (Table 10).

Table 9. Reagents and volumes used in reverse transcriptase (2,5 times the volumes in the protocol).

Reagents	Volume per sample
10x RT buffer	5 µL
25x dNTP mix	2 µL
10x RT Random primers	5 µL
Multiscribe reverse transcriptase	2,5 µL
Nuclease free H_2O	10,5 µL

Table 10. Thermal program for cDNA synthesis by reverse transcriptase.

Steps	Temperature	Time
Step 1	25°C	10 minutes
Step 2	37°C	120 minutes
Step 3	85°C	5 minutes
Step 4	4°C	Indefinite

Amplification by qRT-PCR

qRT-PCR amplify target cDNA sequences, and was used for measuring mRNA expression levels. To amplify cDNA for measuring mRNA expression, RNA had to be converted from RNA to cDNA[5]. qRT-PCR was then performed using the cDNA from the cDNA synthesis, and in this thesis TaqMan assays were used. The amount of amplified cDNA is measured by fluorescent, and shown with an exponential curve corresponding to the template present in a given sample. Fluorescent is either added in dsDNA-binding dye, or attached to probes in the amplification. In this thesis, fluorescent probes were used in the form of TaqMan assays, following manufacturer's protocol.

The principle of this method is to add a DNA binding probe containing reporter (R) in the 5' end and quencher (Q) in the 3' end. While attached closely the quencher inhibits the reporter from expressing fluorescents. When the amplification reaches the probe the DNA-polymerase exonuclease activity destroys the probe and the free reporter expresses fluorescents (Figure 10). The target template will increase exponentially, and be visualized by the correspondent increase of fluorescents detected in the sample. A low amount of template present from the start will result in a late exponential phase, and an early exponential phase will occur if a high amount of template is present, this number is expressed as the CT value (Figure 11).

[5] Described in section 3.3.2 cDNA synthesis

In addition to the assays for the target sequence, here used *F5*-Assay, *IL6*-Assay and *IL8*-Assay, an endogenous control was used for regulation of any variations in template input, or reverse transcription efficiency. In this thesis both PMM1 and GAPD were used as endogenous controls. PMM1 is affected by doxorubicin, and GAPDH was used when cells had been treated with doxorubicin, GAPDH had been shown previously in our lab to not be affected by doxorubicin treatment.

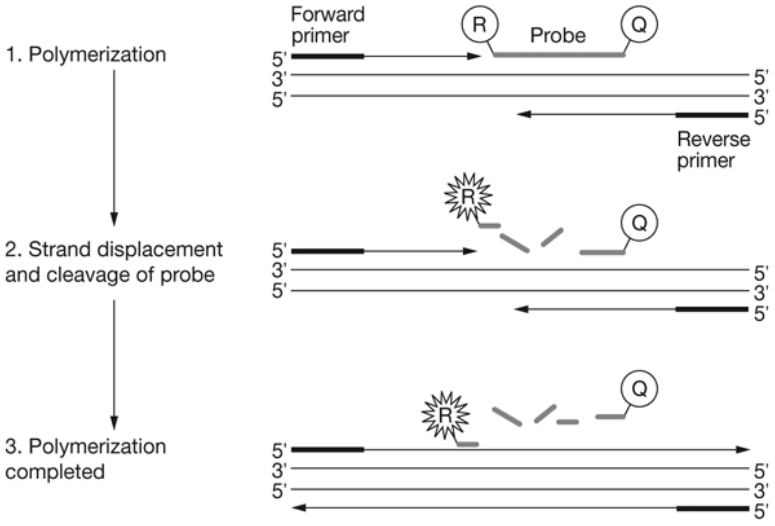

Figure 10. Principle of the TaqMan technique. A probe with both reporter (R) and quencher (Q) attached, will bind to DNA. Primers are added, and polymerase with 5' endonuclease activity cleaves the probe during amplification. The R and Q are now separated from each other and the Q is no longer inhibiting the fluorescent from the R, resulting in a detectable signal (Paik et al. 2005).

Table 11. Composition of reagents for qRT-PCR for FV, IL8 or IL6 overexpression. The reagents are shown for one sample in a 384-well plate.

Reagents	**Volume**
TaqMan® Gene expression master mix	5,0 µL
Assay (*F5*, *IL6*, *IL8*, PMM1 or GAPDH)	0,5 µL
cDNA (for NTC nuclease free H_2O)	4,5 µL
Total volume	10 µL

TaqMan® Gene Expression Master Mix was in used in this thesis, and manufacturer's protocol was followed. All reactions were prepared from the cDNA synthesis samples, and diluted to contain between 40-200ng of template. All samples within the same experiment contained the same amount of cDNA input. cDNA dilutions were mixed with TaqMan master mix containing the target assay (Table 11) and endogenous control in a 96-well plate.

Endogenous control should have an equal expression in all samples, and not be affected by any stimuli. In addition, a non-template control (NTC) was added for each assay used to verify a contamination free assay, the NTC was therefore added H_2O instead of cDNA. The samples were added in triplicates, 10 µM per well in a 384-well plate. The samples were analysed using the QuantaStudio 12k Flex.

Analysing changes in gene expressions were measured relative to the control, using threshold in the exponential phase, the CT value, to calculate the relative quantity ($RQ=2^{-\Delta\Delta CT}$) (Figure 11).

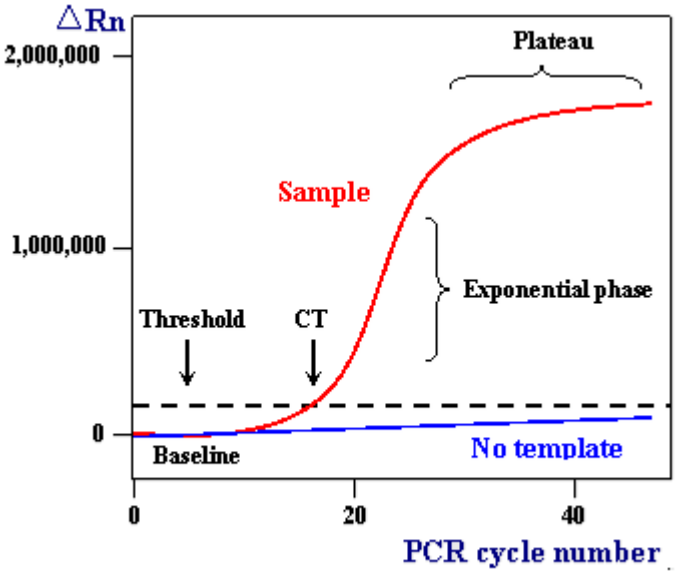

Figure 11. qRT-PCR exponential detectable curve. Signals from the reporter from the TaqMan assay, will be detected and increase exponentially with the cDNA replication. The exponential curve shows the amount of template present by the CT value. The CT value is based on value at the X-axis, where the exponential curve crosses the threshold line (showed in dotted line). The lower the CT number, the higher concentration of template present (Dragoni).

3.3.3 DNA sequencing by sanger sequencing

Sanger sequencing is a sequencing technique based on dideoxynucleotide (ddNTP) termination. The ddNTP are nucleotides lacking the 3' hydroxyl group, which terminates the amplification process. At the end of each ddNTP a fluorescence dye is attached, and the four different nucleotides is detectable by capillary electrophoreses. Together with ddNTP we added deoxynucleotides (dNTP), a primer and DNA template in one sample. DNA fragments of different lengths are produced, because of the random adding of ddNTP and dNTP (Figure 12).

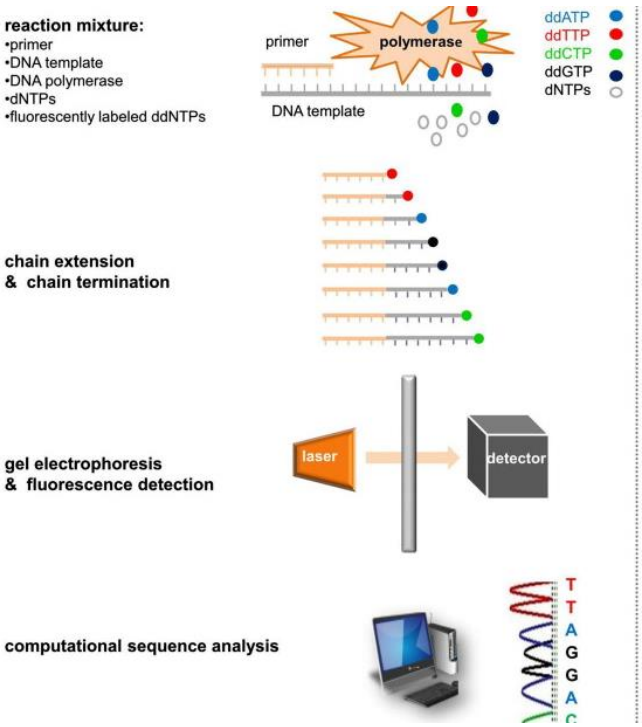

Figure 12. Sanger sequencing technique by capillary electrophoresis. Primer, DNA template, DNA polymerase, dNTPs and ddNTPs are added in one PCR sample. A sequencing PCR is performed, where chain termination will happen for each ddNTP attached. Resulting in fragments of different lengths containing a fluorescent labeled ddNTP in each end. After samples are cleaned, the DNA fragments will be separated and detected by a laser in capillary electrophoresis. The results is analyzed by a computer (Chapter 2 - Techniques for Oral Microbiology 2015).

Sanger sequencing was in this thesis performed to confirm the pcDNA5/FRT-FV contained the entire *F5* gene, and if there were any new mutations of significance. To be able to cover the entire gene 20 different sequencing primers were used[6], in 20 different reactions (Table 12). BigDye Terminator v.3.1 cycle sequencing kit was used, and followed by manufacturer's protocol. In short, plasmids were diluted to be within the end concentration ranging from 110-200ng, and primers diluted to 3,2pmol. Reactions were added in a 96-well plate and amplified by Veriti96 Well Thermal Cycler. After PCR amplification, the samples were cleaned by CleanSEQ[7], according to manufacturer's protocol, and analysed with capillary gel electrophoresis by ABI 3730 sequencing analyser. The sequences were aligned and compared to the *F5* reference sequence (NM_000130.4) using the software DNA Sequence Analysis software v5.1.

[6] Primers listed in Table S1 in appendix 1
[7] Described in section 3.3.4

Table 12. Reagents used in one reaction of sequencing. One reaction per primer was used, for each plasmid, in total 20 reactions.

Reagents	Volume x1 reaction
Nuclease free H_2O	5,75 µL
Plasmid (pcDNA5/FRT-FV)	1 µL
5x Sequencing buffer	2 µL
Primer	1 µL
Big Dye	0,2 µL
Total volume	10 µL

3.3.4. DNA purification

Purification of DNA was performed after sequencing PCR, and before sequence analysing by capillary electrophoresis. After sequencing PCR, the samples still contain all reagents including un-used dNTP and ddNTP, these reagents cause noise in the capillary electrophoresis. A clear PCR sample is needed to perform a successful capillary electrophoresis, with clear and high signal intensity. In this thesis Agencourt® CleanSEQ – Dye removal kit was used and performed on a BioMek FX robot (Figure 13). The procedure was conducted according to manufacturer's protocol.

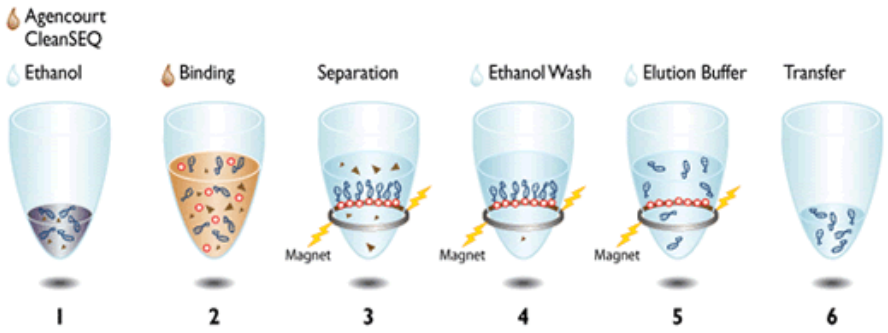

Figure 13. Illustrating the Agencourt® CleanSEQ principle, performed by a BioMek FX robot. 1) Ethanol and Agencourt CleanSEQ reagent is added to the sample. 2) The DNA sequence binds to a magnetic bead, to be able to separate them from other reagents. 3) Plate is being transferred to a magnet plate where magnetic beads bind to the outside magnet. 4) Samples are being washed clean for all other reagents, not binding to the magnet. 5) An elution buffer is added, releasing the DNA sequence from the magnetic bead and into the buffer. 6) The complete cleaned sample, containing only DNA sequences, is being transferred to a clean plate for further analyzing by gel electrophoresis (BackmanCoulter).

3.3.5 *In vitro* mutagenesis

Site-directed mutagenesis is a method used for making changes to a DNA sequence. This could be used either to add changes, or to correct mutations, in our case it was used to correct mutations occurring during long-range PCR. The QuickChange II XL Site-directed mutagenesis kit was in this thesis used according to manufacturer's protocol. Primers for the correction of mutations were designed according to the specifications in the kit protocol (Table 13).

Site-Directed mutagenesis was performed by synthesis of the pcDNA5/FRT-FV plasmid using the primers designed to correct the mutations, both forward and reverse primer. The reaction mix was created according to the manufacturer's protocol (Table 15). The DNA polymerase used in this process is *PfuUltra* HF, a very specific DNA polymerase used together with the Quick Solution, for a correct replication of large plasmids. The reactions were run on thermal cycles described in Table 14.

After synthesizing of the plasmid, the template plasmid was destructed so only the corrected version is present in the sample. The destruction is accomplished by the adding of 1 µL *Dpn1* restriction enzyme to each sample after mutagenesis PCR was complete. The *Dpn1* was activated at 37ºC for 1 hour. *Dpn1* is a restriction enzyme targeting only methylated DNA, resulting in only degradation of the template plasmid, which is the only one methylated. The newly synthesized plasmid is not methylated, and is therefore not digested by the *Dpn1*.

Tabell 13. Site-directed mutagenesis primer forward and reverse.

Primer	Primer sequence 5'-3'
FWD primer c.189 mutation	CTGTAACTTCCTTTAAGAAAATTGTCTACAGAGAGTATGAACC
REV primer c.189 mutation	GGTTCATACTCTCTGTAGACAATTTTCTTAAAGGAAGTTACAG

Tabell 14. Cycling parameters for Site-directed mutagenesis run on the Veriti 96 Well Thermal Cycler.

Temperature	Time	Stage	Cycles
95°C	1 minute	Stage 1	x1
95°C	50 seconds	Stage 2	x18
60°C	50 seconds		
68°C	12 minutes		
68°C	7 minutes	Stage 3	x1
4°C	Indefinite	end	x1

Tabell 15. Reagents and volumes for the Site-directed mutagenesis reaction.

Reagents	Sample x1 reaction
10x reaction buffer	5 µL
dsDNA template (plasmid) (10ng)	1 µL
Forward primer (125ng)	1 µL
Reverse primer (125ng)	1 µL
dNTP mix	1 µL
Quick Solution	3 µL
Nuclease free H_2O	38 µL
Total volume	50 µL

3.3.6 Measure DNA and RNA purity

Spectrophotometers are instruments using absorption of UV light to quantify and determine purity of samples including DNA, RNA and proteins. In this thesis NanoDrop® ND-1000 was used to quantify and determine purity of DNA and RNA samples. Quantity of the samples were measured at the nucleic acid absorbance at 260nm and purity 260/280nm, where proteins absorbs at 280nm.

3.3.7 Isolation of RNA

Cell lysed in RNAqueous from harvested cells were purified and isolated using RNAqueous® Kit, following manufacturer's protocol. For the best possible binding of RNA to the filter in the spin column, the cell lysate was diluted in 64% ethanol buffer and centrifuged at 12000 rpm for 30 seconds. Purifications of the RNA followed in three washing steps with solutions containing ethanol, these eluates the contaminants from the filter. One final centrifugation was carried out to remove any ethanol left from the washing steps. Elution of RNA from the filter was conducted with the use of pre-heated (70°C) elution solution. The elution was performed twice, once with 50 µL and the second time with 40 µL. Isolated RNA was placed directly on ice before stored at -20°C.

3.4 Cell techniques

3.4.1 Breast cancer cell lines

MDA-MB-436

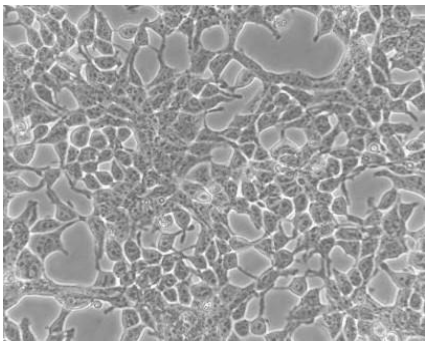

Figure 14. MDA-MB-436 during culturing.

The triple negative basal adenocarcinoma MDA-MB-436 cell line (Figure 14), was used in expression and growth studies. MDA-MB-436 cells were cultured in Nunc™ Cell Culture treated bottles, and 10% Fetal Bovine Serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM) was used during studies and culturing. In growth studies MDA-MB-436 cells were seeded out in a 96-well plate and in 12-well plate for expression studies. MDA-MB-436 cells are mutant for the p53 gene, and have a low endogenous expression of FV. MDA-MB-436 are known to be resistant for treatment with doxorubicin.

MDA-MB-231

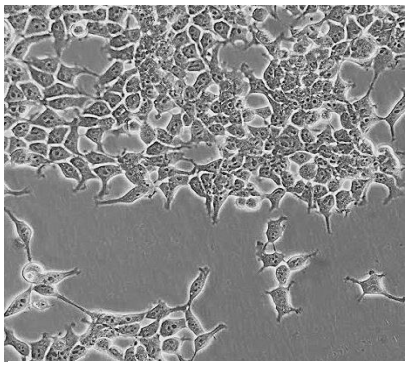

Figure 15. MDA-MB-231 during culturing.

The triple negative basal metastatic adenocarcinoma MDA-MB-231 cell line (Figure 15), was used in expression studies and growth studies. MDA-MB-231 cells were cultured in Nunc™ Cell Culture treated bottles, and 10% FBS in DMEM growth medium was used during studies and culturing. In expression studies MDA-MB-231 were seeded out in 6- or 12-well plates and in 96-well plates for growth studies. MDA-MB-231 cells are mutant p53 cells, but still express p53, and have a low endogenous expression of FV. MDA-MB-231 cells are known as resistant to treatment with doxorubicin.

MCF-7

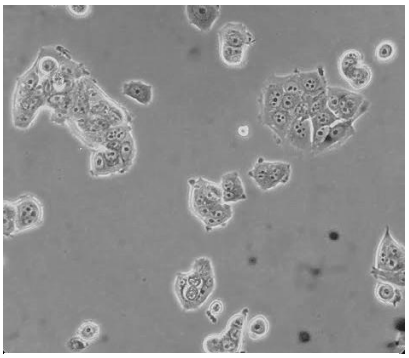

Figure 16. MCF-7 during culturing.

The luminal, ER/PR+ and HER2- metastatic adenocarcinoma MCF-7 cell line (Figure 16), was used in expression studies and growth studies. MCF-7 cells were cultured in Nunc^{TM} Cell Culture treated bottles, and 10% FBS in RPMI 1640 with L-Glutamine was used during studies and culturing. In expression studies MCF-7 were seeded out in either 6 or 12-well plates and a 96-well plate for growth studies. MCF-7 cells are wt for the p53 gene, and have a low endogenous expression of FV. MCF-7 is known to be a sensitive cell line for doxorubicin.

SUM102

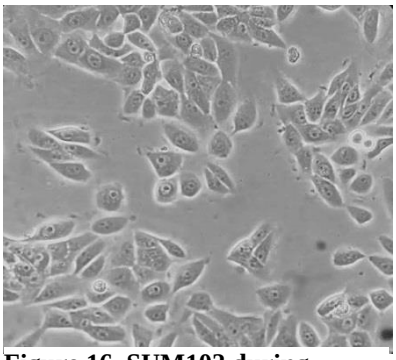

Figure 16. SUM102 during culturing.

The triple negative basal invasive ductal carcinoma SUM102 cell line (Figure 16), was used in expression studies and growth studies. SUM102 cells were cultured in Nunc^{TM} Cell Culture treated bottles, using Serum Free HuMEC with HuMEC Supplement Bovine Pituitary Extract (BPE) during studies and culturing. In expression studies SUM102 was seeded out in either 6 or 12-well plates and a 96-well plate for growth studies. SUM102 cells are p53wt, and have a moderate endogenous expression of FV. SUM102 is known to be sensitive cells for treatment with doxorubicin.

3.4.2 Transfection

Cell transfection is the process when exogenous DNA is transferred into cells, resulting in a genetically modified cell. This enables for studying gene function and products in live cells. There are several different transfection approaches. In this thesis transient transfection was used, incorporating DNA into the cell for expression, but not into its chromosome.

DNA was transfected into the cell by liposome-based gene transfection. In short, DNA is transferred into a liposome, the lipids merge with the cell membrane, and DNA are released into the cell. Lipofectamine Reagent 3000 Protocol was followed during transfection of pcDNA5/FRT-V-wt into the breast cancer cell line MDA-MB-231 (Table 16). A suspension of $1{,}5x10^5$ cells/mL was made with 10% FBS DMEM for seeding out cells, before incubation

at 37°C in 5% CO_2 overnight. After incubation 250 µL containing lipofectamine mix and the plasmid mix was added to each well, and medium was changed after 4-6 hours. Cells were after transfection incubated at 48 hours at 37°C in 5% CO_2 before harvested. When performing this experiment, controls of pMT2-V-wt, pcDNA5/FRT-V and pcDNA5/FRT plasmids were used as controls for the overexpression of FV. All plasmids were transfected in duplicates or triplicates.

Table 16. Reagents and amounts for a 6-well plate of transfection. Amounts are shown for one well.

Reagents	DNA mix	Lipofectamine mix	Cell mix
OptiMEM	125 µL	125 µL	-
Plasmids	2,5 µg	-	-
P3000	5 µL	-	-
Lipofectamine 3000	-	7,5 µL	-
DMEM 10% FCS	-	-	1,5 mL
MDA-MD231	-	-	3,0x10E5

3.4.3 Harvesting of medium and cell lysate

Medium and cell lysate for protein and mRNA expression analyses were harvested for each transfected plasmid. All the medium from each well was collected in tubes for protein expression analysis. Cells were then lysate using RNAqueous for RNA samples, and RIPA solution for protein samples (inhibitor cocktail (x100) in relation inhibitor 1:100 RIPA buffer) and collected in tubes for storage. All samples were placed directly on ice before stored at -4°C or -20°C for short time storage and -80°C for long time storage.

3.5. Chemotherapeutic treatment of breast cancer cells

The chemotherapeutic drug doxorubicin was in this thesis used to investigate response in FV, p53 and cell viability, to treatment in different breast cancer cells.

3.5.2 Breast cancer cells exposed to increased concentrations of doxorubicin

We wanted to investigate how expression of *F5* mRNA was effected by doxorubicin in breast cancer cells. Therefore, four cell lines (SUM102, MCF-7, MDA-MB-231 and MDA-MB-436) were treated with increased concentrations of doxorubicin (0 µM, 0,1 µM, 1 µM and 5 µM).

Cells were seeded out in a 12-well plate and grown for 24 hours, 500 µL of the diluted doxorubicin[8] was then added to each well. Cells were further incubated for 24 hours at 37°C with 5% CO_2, before harvested[9]. RNA from the samples were isolated[10] and analysed by qRT-PCR[11].

SUM102, MCF-7 and MDA-MB-231 cells were also treated with 1 µM doxorubicin concentration and incubated for 24h and 48h. This was performed to see if there was any time dependent effect on doxorubicin-induced *F5* mRNA. The procedure was here performed as explained above. RNA samples were furthermore isolated and analysed by qRT-PCR.

3.5.3 Cell viability during increased concentrations of doxorubicin

To investigate the different breast cancer cells viability to doxorubicin treatment, we used an induced concentration of doxorubicin reaching from 0 µM to 10 µM. All four cell lines were seeded out in 7 replicates into a 96-well plate and incubated for 24 hours at 37°C with 5% CO_2. After 24 hours 50 µL of diluted doxorubicin for each concentration was added in each well, and cells were again incubated for 24 hours. To measure the cell viability after 24 hours incubation, 20 µL of the cell proliferation agent Wst-1 was added to each well. Cells were incubated for another 30 minutes at 37ºC and absorbance was measured by using the VersaMax™ microplate reader.

Measurements on cell viability by Wst-1 is based on the presence of NAD(P)H production in living cells, and a cleavage from tetrazolium salt to a soluble formazan results in colour change. This colour change was measured at Lm1: 450nm and Lm2: 745nm by the VersaMax™ microplate reader, and analysed using SoftMax Pro6.4 software. Measurements on viability were performed using Lm2 as reference and calculating results by Lm1 – Lm2.

3.5.4. p53 inhibited breast cancer cells treated with doxorubicin

In this thesis, we wanted to investigate the effect of p53 on *F5* mRNA expression when cells were treated with doxorubicin. Therefore, inhibition of the p53 gene was performed on three p53 expressing breast cancer cells (SUM102, MCF-7 and MDA-MB-231), using a dilution of Pifithrin-α (PFT-α).

[8] Described in section 3.5.1
[9] Described in section 3.4.3
[10] Described in section 3.3.7
11 Described in section 3.3.2 Amplification by qRT-PCR

Cells were seeded out in 12-well plates and grown for 24h, at 37°C with 5% CO_2. After 24 hours incubation, the medium was removed from the cells and 1 mL new medium solutions were added to each cell type (Table 17). PFT-α was first diluted to 3060 µM, before it was further used in the solutions, each solution was made from a combination of PFT-α, DMSO and cell specific medium. Solutions were made specific for each cell type, with its cell specific medium for culturing[12]. For each cell type five different combinations were made:

1. Control (DMSO and PBS added)
2. Only doxorubicin treated (DMSO and doxorubicin added)
3. Only p53 inhibited (20 µM PFT-α and PBS added)
4. Both p53 inhibited and doxorubicin treated (20 µM PFT-α and doxorubicin added)
5. Both p53 inhibited and doxorubicin treated (30 µM PFT-α and doxorubicin added)

First only DMSO and PFT-α solutions were added to the wells. After a 24 hour incubation with the solutions, 30 µL of doxorubicin from a 34 µM solution (1µM) was added to each well and PBS was added to the control cells and only p53 inhibited cells.

Table 17. Volumes of diluted PFT-α solutions. Dilutions made for one cell line, one was made for each cell line with its cell specific medium.

Solution	Inhibitor/vehicle	Medium	Total volume
DMSO	50 µL DMSO	9,95 mL	10 mL
PFT-α 20 µM	11,7 µL DMSO + 23,3 µL diluted PFT-α	6,965 mL	7 mL
PFT-α 30 µM	20 µL diluted PFT-α	3,980 mL	4 mL

3.6 Protein techniques

3.6.1 Total protein quantification

Total protein was measured on cell lysate harvested from transfected cells. The total protein quantification method measures the total amount of proteins present from the lysate. Measurements were made using the PIERCE® BCA Protein Assay Kit, and manufacturer's protocol was followed. The principle of this analyse is the chelate complex formed by the proteins when Cu^{2+} ions reduces the Cu^{2+} to Cu^{1+} and reacts with the BCA. This reaction results in the reaction turning purple, the higher level of protein the darker the colour. BCA-Cu^{1+} absorbs UV light at 570nm and by measuring the reactions at this absorbing level by

[12] Described in section 3.4.1

VersaMax™ microplate reader. We will get a proportional absorption to the amount of proteins present.

Samples were plated on to a 96-well plate with a flat bottom, 5 µL of each sample and standards were placed in triplicates. A working reagent made from reagent A and reagent B in the kit (BCA) was added, 200 µL to each well. The plate was incubated at 37°C for 30 minutes, after incubation the samples were measured at 570nm.

Analysing of the samples was performed using SoftMax Pro 6.4 software, which calculates the concentration from a standard curve[13]. The standard dilution series for creating this curve was added to the sample-plate, consisting of a series of 2x Albumin constituting six dilutions with concentrations of 0-2mg/mL.

3.6.2. Enzyme-Linked Immunosorbent Assay (ELISA)

To quantify the presence and concentration of specific proteins in a sample we used the enzyme-linked immunosorbent assay (ELISA). There are several different models of ELISA, and in this thesis the sandwich ELISA was used, named by the analyte being kept between to antibodies. One antibody, the primary, is attached to a well in a microplate, the analyte will attach to the antibody, and then the secondary antibody will bind to the analyte. The secondary antibody will by the adding of a substrate produce a product that absorbs light at a specific wave length (Figure 17).

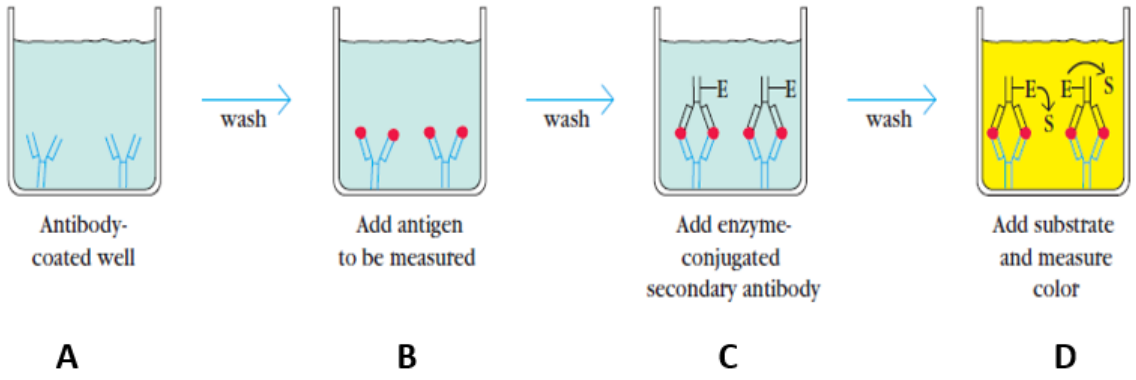

Figure 17. ELISA capture assay «sandwich» illustration. Demonstrating the main steps in ELISA sandwich model. A) The antibody is attached to the microplate and B) binds the target protein. C) the secondary antibody then binds to the protein and D) when substrate is added colour changes in accordance to protein concentration (Giri 2015).

[13] Standard curve displayed in Figure S2 in Appendix 3

In this thesis ELISA was performed to detect the FV protein expressed by the different FV expression plasmids: pMT2-FV-wt, pcDNA5/FRT-FV(c.189/c.2694) and pcDNA5/FRT-FV(c.2694). To find the protein concentration, media and protein lysate samples were used in the ELISA ZYMUTEST FV, which detects human FV antibody. The test was performed according to manufacturer's protocol. The wells in the microplate used were pre-coated with a monoclonal antibody for FV and samples were added directly into the well. In addition, a standard ranging from 0-100%, as well as high and low FV controls were added to the microplate. Samples were incubated at 37°C before adding of the polyclonal secondary antibody with horse-radish-peroxidase (HRP), following a second incubation. To develop colour correlating to the amount of protein present, the substrate tetramethylbenzidine (TMB) with hydrogen peroxide was added to each well. Colour gradually appear, and is stopped by the adding of sulfuric acid after 10 minutes. Absorbance was then measured at 450nm by VersaMax™ microplate reader. From the standards with known concentrations a standard curve was created by SoftMax Pro6.4 software[14]. Concentrations from our samples were determined from this standard curve.

3.6.2 Protein activity measured by Luciferase reporter assay

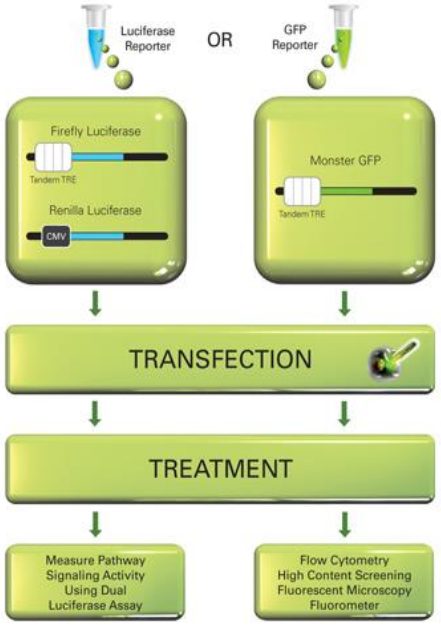

Figure 18. Illustration of the function of Cignal Finder. In this thesis luciferase reporter was used. Specific gene TRE for p53 is located upstream for the luciferase gene. The renilla luciferase plasmid serve as an endogenous control (Qiagen).

In this thesis, the Cignal p53 Pathway Reporter Assay (Qiagen) (Figure 18) was used to measure the p53 activity in MCF-7 and MDA-MB231 cells (3,0x10^4 cells/well). The cells were reverse transfected with the luciferase reporter plasmids with p53 specific transcriptional response elements, and renilla as an endogenous control, as well as the FV plasmids (pcDNA5/FRT, pMT2-FV-wt, pcDNA5/FRT-FV(c.189/c.2694) and pcDNA5/FRT-FV(c.2694)), or treated with doxorubicin/PFT-α 24 hours after the transfection. Protocol was followed as described by the manufacturer. 48 hours after transfection, the cells were lysed in 20µl 1xPLB for use in Dual-Luciferase® reporter Assay System (Promega).

[14] Shown in Figure S3 in Appendix 4

3.7 Statistics

The statistics in this thesis, calculating differences in significance, was performed by a two-sample T-test. Significance of the p-values were determined by significant $p<0,05$, or non-significant $p>0,05$. The p-values in this thesis will be defined in figures as $*p<0,05$ and $**p<0,001$. Some of the experiments conducted here had in addition a low number of repeated experiments and parallels, especially for the FV inhibition experiment that were only performed twice with few parallels. The statistics form this thesis should therefore be interpreted with care in drawing definite conclusions.

4. Results

4.1 Overexpression of FV in MDA-MB-231 cells

To study the functional effects of FV in breast cancer, we first needed to overexpress FV in the cells. In order to gain this overexpression, we created a new FV expression construct. This process required a plasmid which could easily be transfected and create stable gene overexpression in eukaryotic cell lines. We possessed a commercially available vector containing the full-length cDNA of the *F5* gene (pMT2-FV-wt), however, we had limited knowledge about the features of this vector. In addition, we lacked an empty version of the vector. We therefore decided to subclone the *F5* cDNA into another expression vector (pcDNA5/FRT) of which features were known. Moreover, this vector was compatible with the Flp-In™ Complete System, that allows integration and expression of a gene in mammalian cells at a specific genomic location.

4.1.2 Construction of a FV overexpression plasmid

Amplification and restriction enzyme digestion of F5 cDNA

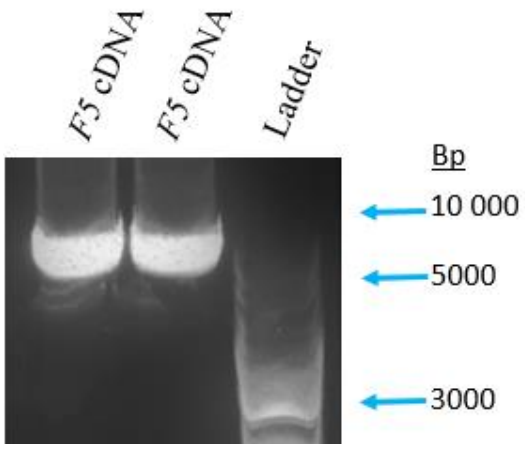

Figure 19. Agarose gel electrophoresis of long-range PCR of F5 cDNA fragments. Fragments of F5 cDNA was amplified by long-range PCR using pMT2-FV-wt as template. F5 cDNA is 7000bp in size, appearing approximately between the 10 000 and 5000bp marks.

In order to subclone the *F5* cDNA from pMT2-FV-wt to the pcDNA5/FRT vector, the *F5* cDNA needed first to be isolated from pMT2-FV-wt. Since there were no common restriction sites that could be used, the *F5* cDNA in pMT2-FV-wt was amplified with long-range PCR using a primer pair based on the *F5* RNA sequence (NM_000130). To create a *F5* fragment with restriction sites in each end that were compatible with the recipient vector, the primers were tailed with recognition sites for the restriction enzymes *Hind III* and *Not I*. Successful amplification of the *F5* cDNA fragment was confirmed by gel electrophoresis (Figure 19). *F5* cDNA was purified directly from the PCR reaction to avoid loss of DNA during gel purification. The purified *F5* cDNA fragment was

digested with *Hind III* and *Not I* restriction enzymes, separated by gel electrophoresis, and finally isolated from the gel for further use.

Preparation of the pcDNA5/FRT vector

To be able to ligate the *F5* cDNA fragment (isolated above) into the pcDNA5/FRT vector, the vector needed to be linearized. The pcDNA5/FRT was digested with restriction enzymes *Hind III* and *Not I* to generate compatible ends, which were capable of being ligated with the *F5* cDNA fragment. Gel electrophoresis was run to separate the linearized vector from the undigested vector and the short DNA fragments resulting from the digestion. Successful digestion of the vector was confirmed, and thereby the linearized pcDNA5/FRT vector (Figure 20) was isolated from the gel and purified for further use.

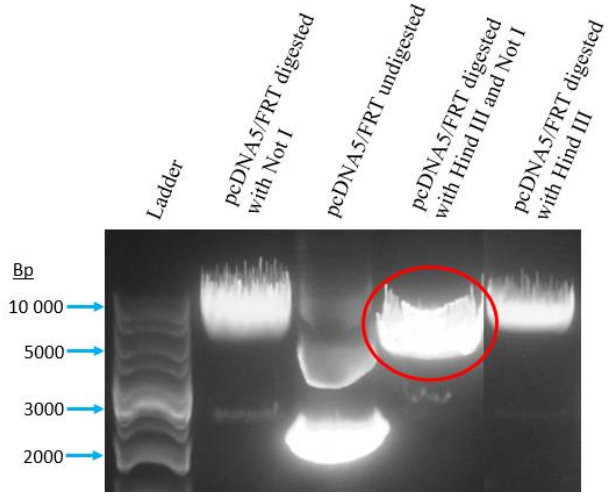

Figure 20. Agarose gel electrophoresis of pcDNA5/FRT before and after enzyme digestion. Vector digested with *Hind III* and *Not I* (marked in red circle) was extracted from the gel and purified.

Ligation of F5 cDNA and pcDNA/FRT vector

The digested *F5* cDNA fragment and the digested pcDNA5/FRT vector were ligated to form a new functional FV expression plasmid. The newly created plasmid, hereafter named pcDNA5/FRT-FV, is a large plasmid consisting of 11597bp in total (Figure 21).

To verify that the *F5* cDNA had been successfully ligated into the pcDNA5/FRT vector, the plasmid was digested with *Hind III* and separated by gel electrophoresis (Figure 22). The digestion clearly demonstrated the size difference between the supercoiled and the linearized plasmid. The newly created pcDNA5/FRT-FV plasmid showed the expected size of approximately 11kb and the vector alone was about 5kb (Figure 22).

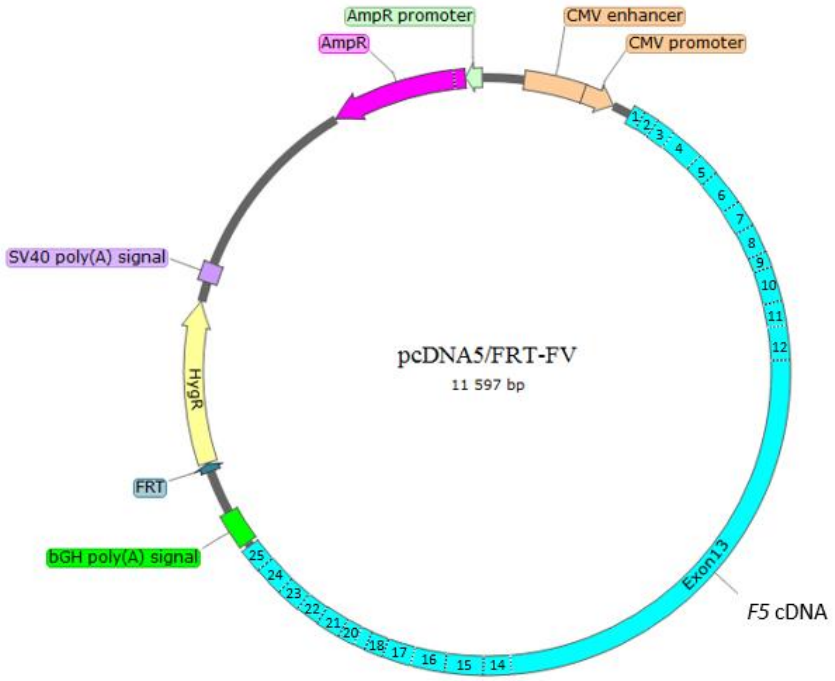

Figure 21. Schematic illustration of the pcDNA5/FRT-FV expression plasmid. The *F5* cDNA with exons shown in blue and insert location in the pcDNA5/FRT vector. Important areas for cloning and transformation in vector pcDNA5/FRT are shown with highlighted arrows.

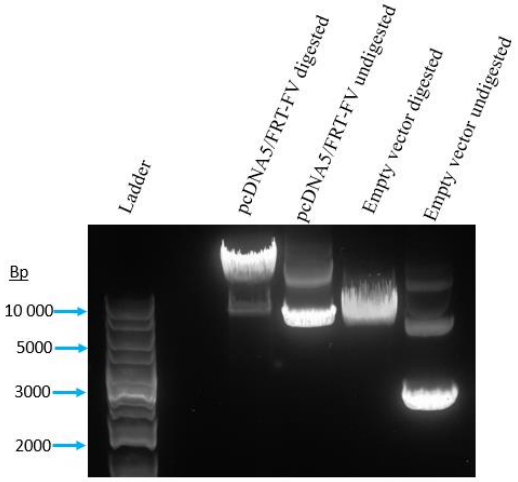

Figure 22. Digested and undigested pcDNA5/FRT-FV and empty vector (pcDNA5/FRT). pcDNA5/FRT-FV and empty vector (pcDNA5/FRT) were digested with restriction enzyme *Hind III*.

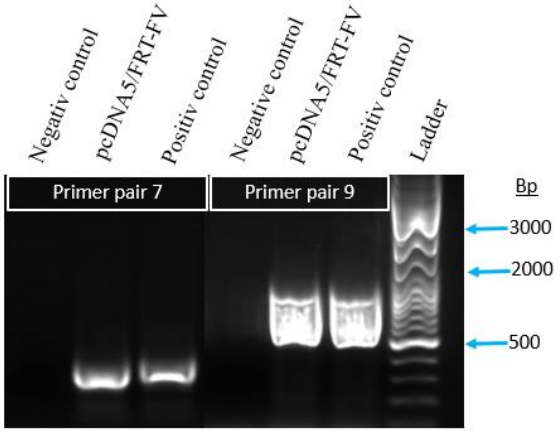

Figure 23. Control PCR to confirm vector incorporation of *F5* cDNA. Control PCR of the pcDNA5/FRT-FV was performed using two *F5* primer pairs. Empty vector (pcDNA5/FRT) was used as negative control and pMT2-FV was used as positive control.

To further confirm the incorporation of *F5* cDNA in the vector, we performed a PCR with two primer pairs within the *F5* gene, amplifying only small DNA fragments of the *F5* gene. The presence of *F5* cDNA was demonstrated by gel electrophoresis where a band of the expected size of approximately 500bp (primer 9) and 300bp (primer 7) appeared in both pcDNA5/FRT-FV, and in the positive control (pMT2-FV-wt), but not the empty vector

control (Figure 23). The newly created pcDNA5/FRT-FV plasmid was further amplified by transformation into competent *E.coli*. Several colonies were isolated for further verification by DNA sequencing.

4.1.2. Sequencing and *in vitro* mutagenesis of the FV plasmid

To confirm the correct *F5* cDNA sequence after the subcloning, plasmids from several bacterial colonies were sequenced and aligned against the reference sequence (NM_000130). It turned out that for all sequenced plasmids mutations had been introduced during the subcloning procedure. The plasmid with fewest mutations kept two new mutations (Figure 24), one in position c.189 (segment A1, exon 2) and the second in position c. 2694 (segment B, exon 13). Mutation in position c.189 changed from an A to G and mutation in position c.2694 changed from a T to G (Figure 25). The positions of the mutations were retrieved from the Alamut database[15]. Both mutations were synonymous. However, according to Human Splicing Finder the c.189, mutation was likely to affect splicing. Nevertheless, this plasmid was collected for further use, as it held the lowest rate of new mutations. Hence, it was collected with the intention of correcting the mutations.

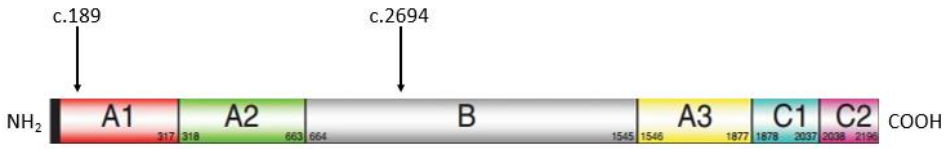

Figure 24. Schematic illustration of the *F5* domain structure. Domain size and position shown in coloured boxes. The positions of mutations c.189 and c.2694 are marked with arrows (modified from Asselta et al 2006).

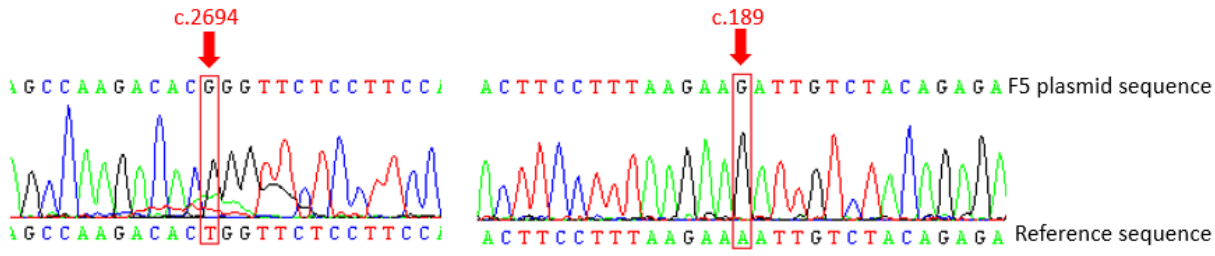

Figure 25. Illustrating mutations in position c.2694 and c.189 in the *F5* sequence. The synonymous mutation c.2694 exchanged a T with a G. The mutation c.189 exchanged an A with a G, also a synonymous mutation.

[15] Position numbers of the mutations retrieved from Alamut, was measured after A, in ATG (ref.seq. NM_000130.4)

The mutation c.2694 was positioned in a repetitive area in exon 13, therefore constructing a specific primer within the boundaries for mutagenesis showed to be impossible. However, as this mutation was synonymous with no predicted effect on splicing, it was considered not to be of essential functional importance. Eventually, only the mutation in c.189 was corrected. To correct this mutation *in vitro*, mutagenesis was performed on the pcDNA5/FRT-FV (from now addressed as pcDNA5/FRT-FV(c.189/c.2694)) to correct the mutation in position c.189 from G to A. After the mutagenesis the corrected plasmid (from now addressed as pcDNA5/FRT-FV(c.2694)) was re-sequenced, and the correction of the mutation in position c.189 was proven successful (Figure 26).

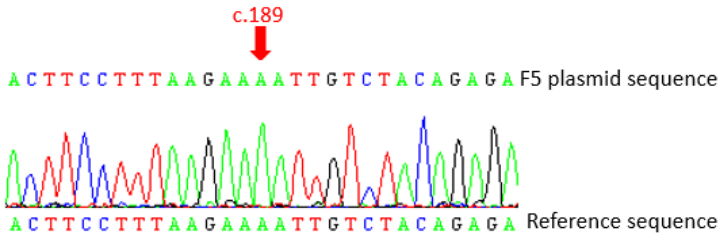

Figure 26. Illustration of the corrected mutation in c.189 after *in vitro* mutagenesis. After *in vitro* mutagenesis of mutation c.189 was performed in pcDNA5/FRT-FV(c.189/c.2694), mutation in position c.189 was confirmed corrected from a G to A by sequencing.

4.1.4 Transfection of the FV constructs in MDA-MB-231 cells

The creation of pcDNA5/FRT-FV(c.2694) was conducted to obtain a functional plasmid showing overexpression of FV for future studies in eukaryotic cells. Thereby, pcDNA5/FRT-FV(c.2694) was transfected into MDA-MB-231 cells to test its capacity to overexpress FV. FV overexpression was analysed at both the mRNA and the protein level.

The *F5* mRNA expression from the pcDNA5/FRT-FV(c.189/c.2694) plasmid was 4000-fold higher than the empty vector, but 4-fold lower when compared to the pMT2-FV-wt plasmid (Figure 27). However, after correction of the c.189 mutation (pcDNA5/FRT-FV(c.2694)) the *F5* mRNA expression level increased 3-fold compared to the pcDNA5/FRT-FV(c.189/c.2694) (Figure 27).

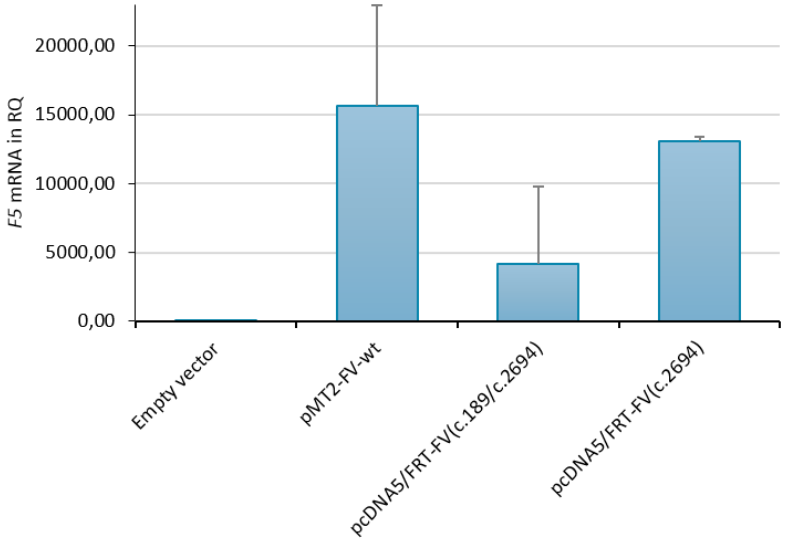

Figure 27. *F5* mRNA expression levels in MDA-MB-231 cells transfected with empty vector (pcDNA5/FRT), pMT2-FV-wt, pcDNA5/FRT-FV(c.189/c.2694) and pcDNA5/FRT-FV(c.2694). Cells were harvested 48 hours after transfection and mRNA were measured using qRT-PCR and normalized against PMM1 as endogenous control. Results are expressed as RQ values compared to the empty vector. Mean values (n=5) + SD from three independent experiments were shown.

FV ELISA was used to confirm the overexpression of FV at the protein level. FV protein levels in media from cells transfected with pMT2-FV-wt, were 5-fold higher in secretion of FV protein than cells transfected with pcDNA5/FRT-FV(c.189/c.2694) (Figure 28). Cells transfected with pcDNA5/FRT-FV(c.2694) corrected for mutation c.189, showed a 1,5-fold increase in FV protein levels compared to pcDNA5/FRT-FV(c.189/c.2694) (Figure 28).

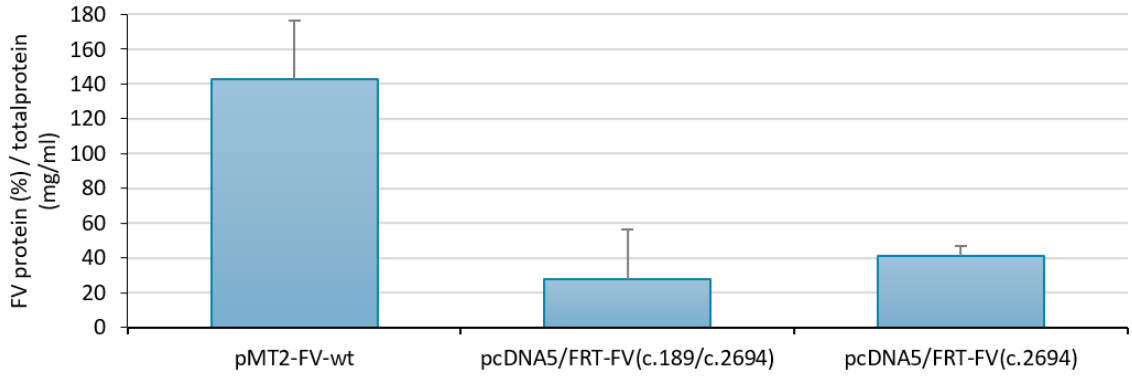

Figure 28. FV protein levels in medium from MDA-MB-231 cells transfected with pMT2-FV-wt, pcDNA5/FRT-FV(c.189/c.2694), and pcDNA5/FRT-FV(c.2694). Cells were harvested 48 hours after transfection. FV protein levels were measured by ELISA and corrected for total protein in lysate. Mean values (n=4) + SD from two independent experiments are shown.

4.1.5 Functional effects of FV in breast cancer cells

Effect on IL6 and IL8

Previous findings in the group suggest a relation between FV and immune response. Hence, it was of interest to see whether there was any effect of FV overexpression on the expression of the inflammatory markers interleukin (*IL*) 6 (*IL6*) and *IL8*, in MDA-MB-231 breast cancer cells.

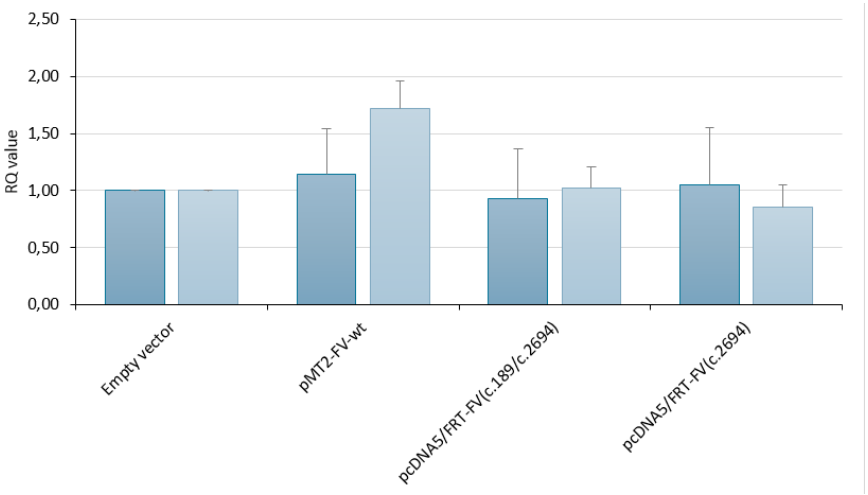

Figure 29. mRNA expression of *IL6* and *IL8* in FV overexpressing MDA-MB-231 cells. Cells were transfected with, empty vector (control), pcDNA5/FRT-FV(c.189/c.2694) and pcDNA5/FRT-FV(c.2694). Cells were harvested 48 hours after transfection and mRNA expression of *IL6* and *IL8* were measured using qRT-PCR and normalized against PMM1 as endogenous control. Results are expressed as RQ values compared to the empty vector. Mean values (n=5) + SD from three independent experiments are shown.

In MDA-MB-231 cells, the pMT2-FV-wt resulted in a 2-fold increase in *IL8* expression compared to control cells, while no change in *IL6* expression was observed (Figure 29). The pcDNA5/FRT-FV(c.189/c.2694) and the pcDNA5/FRT-FV(c.2694) did not significantly affect the expression of either *IL6* or *IL8* (Figure 29).

Effect on p53 activity

To investigate if FV overexpression had any effect on p53 activity in breast cancer cells, a p53 activity assay (Cignal Finder) was performed in breast cancer cells transfected with the different FV expression plasmids. Cells with different p53 status were used, MDA-MB-231 a p53 mutant cell type and MCF-7 a p53wt cell type. Interestingly, p53 activity was also observed in the p53 mutant MDA-MB-231 cells.

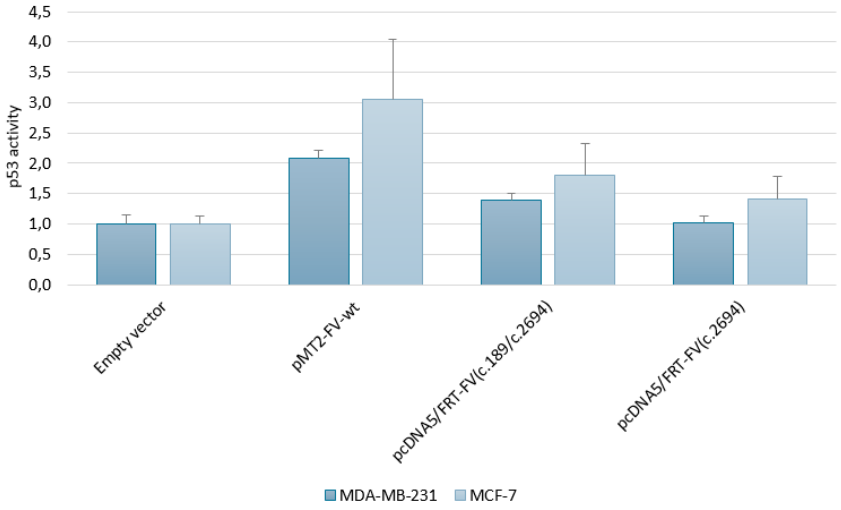

Figure 30. p53 activity in FV overexpressing MDA-MB-231 and MCF-7 cells. p53 activity was measured with Cignal Finder in cells transfected with empty vector, pMT2-FV-wt, pcDNA5/FRT-FV(c.189/c.2694) and pcDNA5/FRT-FV(c.2694). All values were compared to empty vector. Mean values (n=4) + SD from one experiment are shown.

Consistent for both cell lines, the pMT2-FV-wt plasmid showed the most prominent effect on p53 activity with a 2 and 3-fold increased activity compared to control cells for MDA-MB-231 and MCF-7, respectively (Figure 30). In contrast, the pcDNA5/FRT(c.189/c.2694) showed only a 1,4 and a 1,8-fold increased p53 activity, respectively, for the two cell lines (Figure 30). Correction of the c.189 mutation (pcDNA5/FRT-FV(c.2694)) led to a decrease in p53 activity. For this plasmid, no change in p53 activity was observed in MDA-MB-231, while only a 1,4-fold increased activity, corresponding to a 2-fold reduction in p53 activity compared to pMT2-FV-wt, was observed in MCF-7 cells (Figure 30).

4.2 Effect of doxorubicin in breast cancer cell lines

4.2.1 Dose and time dependent effect of doxorubicin on *F5* mRNA in breast cancer cell lines

The influence of chemotherapy induced coagulation activity in cancer patients is well known. However, the molecular mechanisms behind this are not yet fully understood. To investigate if this commonly used agent affects *F5* mRNA expression, four different breast cancer cells were treated with the chemotherapeutic agent doxorubicin in a dose and time dependent manner. Doxorubicin is known to affect p53 activity. We therefore selected cell lines with different p53 status to investigate if any potential effect on *F5* involves p53. To better understand the functional role of FV in cancer, it is important to know how *F5* mRNA expression is regulated in different conditions, including treatment situations.

The breast cancer cell lines SUM102 (p53wt), MCF-7 (p53wt) and MDA-MB-436 (p53 mutant) were treated with different concentrations of doxorubicin, while MDA-MB-231 (p53 mutant) was treated with only 1 µM.

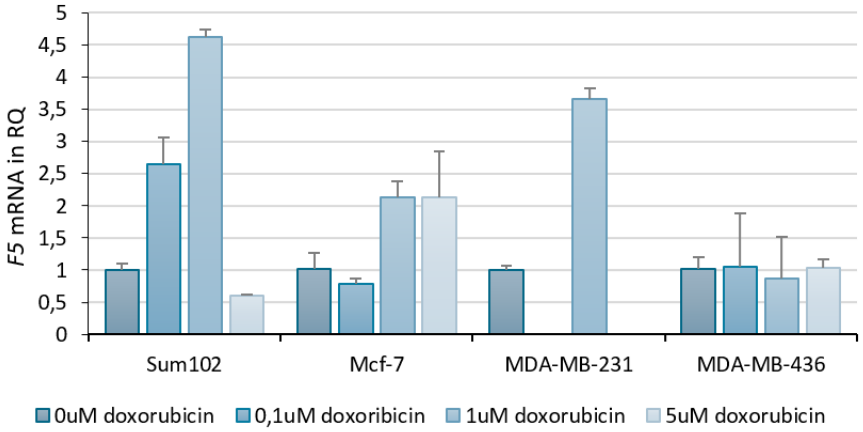

Figure 31. *F5* mRNA expression in SUM102, MCF-7, MDA-MB-231 and MDA-MB-436 cells treated with different concentrations of doxorubicin. Cells were harvested 24 hours after doxorubicin treatment and *F5* expression was measured using qRT-PCR and normalized against GAPDH as endogenous control. Results are expressed as RQ values compared to untreated cells (0 µM). Mean values (n=5) + SD from one experiment are shown.

Treatment with doxorubicin gave an increase of *F5* mRNA expression in all cell lines, except MDA-MB-436 (Figure 31). There was no increase of *F5* expression at the highest dose of doxorubicin (5 µM), which could be due to the toxicity of doxorubicin at this high dose. SUM102 showed a dose response and the highest *F5* expression of all the cell lines with a 5-fold increase at 1 µM. While MDA-MB-231 followed with 4-fold increase at 1 µM, and MCF-7 with a 2-fold increase of *F5* expression. No significant change in *F5* mRNA expression was observed in the MDA-MB-436 cells at any dose of doxorubicin.

To test if the effect of doxorubicin on *F5* expression was time dependent, we also treated the three cells showing an effect with doxorubicin for 48 hours, and compared it to the 24 hour treatment at a dose of 1 µM.

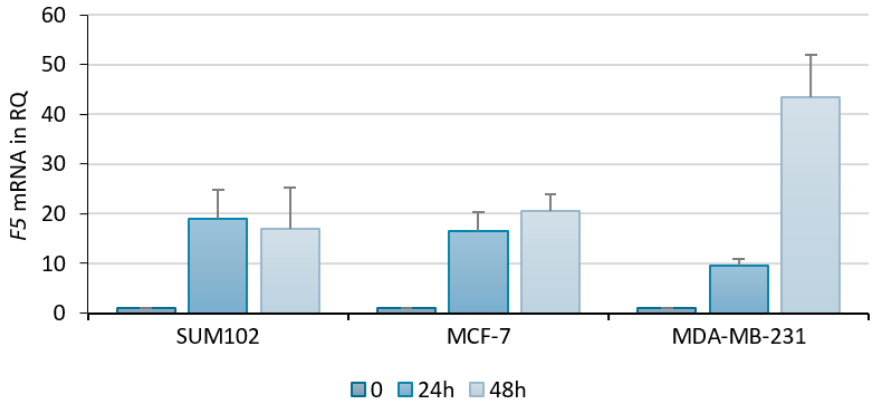

Figure 32. *F5* mRNA expression in SUM102, MCF-7 and MDA-MB-231 after exposure to 1 µM doxorubicin at 24 hours and 48 hours. Cells were harvested 24 and 48 hours after treatment with 1 µM doxorubicin and *F5* expression was measured using qRT-PCR and normalized against GAPDH as endogenous control. Results are expressed as RQ values compared to untreated cells (0 hours). Mean values (n=6) + SD from three independent experiments are shown.

SUM102 and MCF-7 cells showed no further increase in *F5* mRNA expression of significance after 48 hours of doxorubicin treatment (compared to 24 hours). MDA-MB-231 on the other hand, showed a 4-fold increase in *F5* expression when exposed to 1 µM doxorubicin for 48 hours compared to 24 hours (Figure 32). This corresponds to a 43-fold increase in *F5* expression after 48 hours treatment with doxorubicin compared to untreated cells.

4.2.2 The role of p53 in doxorubicin induced *F5* expression

The p53 is a tumour suppressor protein, mainly inducing cell cycle arrest or apoptosis in cancer cells. We wanted to see if p53 was involved in the doxorubicin effect on *F5* expression in the three cell lines that where showing an induced *F5* expression after doxorubicin treatment[16]. We used the p53 inhibitor PFT-α to block p53 in doxorubicin treated MDA-MB-231, SUM102 and MCF-7 cells.

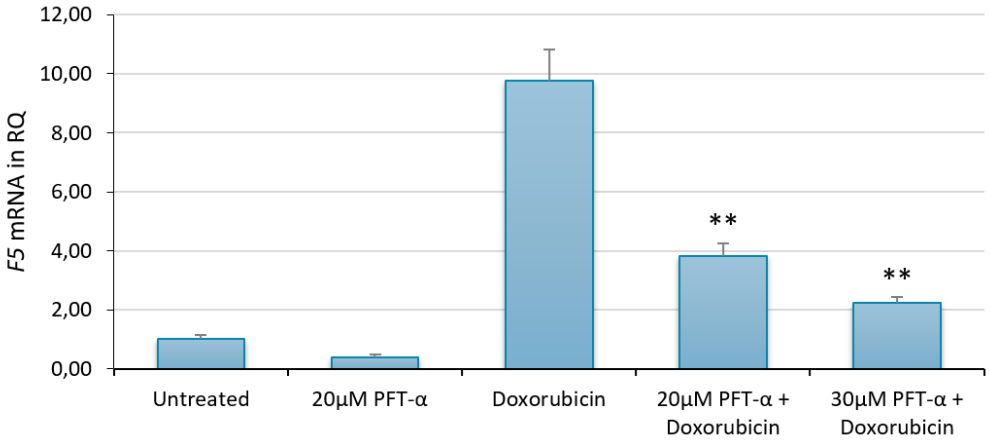

Figure 33. *F5* mRNA expression in MDA-MB-231 cells treated with doxorubicin and PFT-α. MDA-MB-231 cells were untreated (control) or treated with PFT-α 20 µM, 1 µM doxorubicin, PFT-α 20 µM + 1 µM doxorubicin or PFT-α 30 µM + 1 µM doxorubicin. Cells were pre-treated for 2 hours with PFT-α and 24 hours of doxorubicin, before harvested after 48 hours. *F5* expression was measured using qRT-PCR and normalized agains GAPDH as endogenous conrol. Results are expressed as RQ values compared to control cells. Mean values (n=6) + SD from three independent experiments are shown. Significant diffrence ($p \leq 0{,}001$) marked with **.

Upon adding of two different doses of PFT-α, 20 µM (p=0,00000014) and 30 µM (p=0,00000007) in MDA-MB-231 cells before treated with doxorubicin, the induced *F5* mRNA was significantly reduced by 2-4-fold compared to cells treated with doxorubicin only (Figure 33). The figure is displaying an influence of p53 on the doxorubicin-induced expression of *F5* in the MDA-MB-231 cells. Moreover, it shows the treatment of PFT-α alone, has an influence on the *F5* mRNA expression (Figure 33).

[16] Presented in section 4.2.1

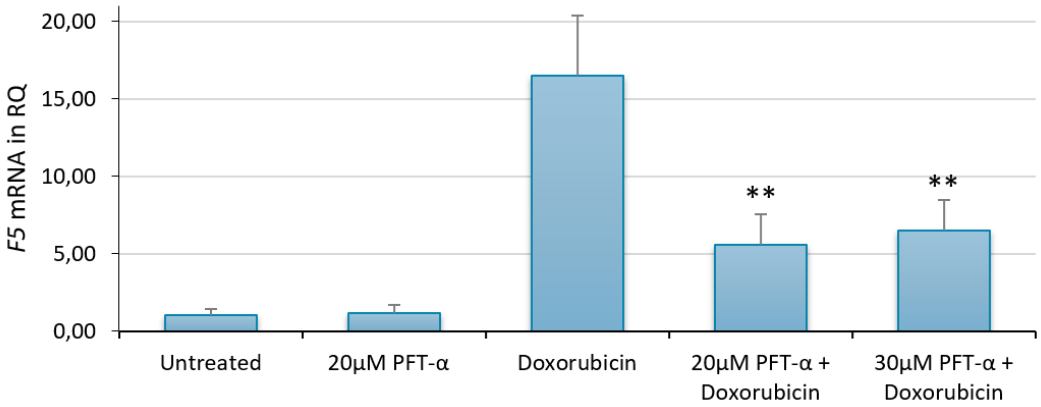

Figure 34. *F5* mRNA expression in MCF-7 cells treated with doxorubicin and PFT-α. MCF-7 cells were untreated (control) or treated with PFT-α 20 µM, 1 µM doxorubicin, PFT-α 20 µM + 1 µM doxorubicin or PFT-α 30 µM + 1 µM doxorubicin. Cells were pre-treated for 2 hours with PFT-α and 24 hours of doxorubicin, before harvested after 48 hours. *F5* expression was measured using qRT-PCR and normalized agains GAPDH as endogenous conrol. Results are expressed as RQ values compared to control cells. Mean values (n=6) + SD from three independent experiments are shown. Significant diffrence ($p \leq 0{,}001$) marked with **.

Similar to MDA-MB-231, adding of two different doses of PFT-α 20 µM (p=0,0001) and 30 µM (p=0,0002) to MCF-7 cells, reduced the induced *F5* mRNA significantly by 2-3-fold compared to cells treated with doxorubicin only (Figure 34). This indicates that p53 influences the doxorubicin-induced expression of *F5* also in the MCF-7 cells.

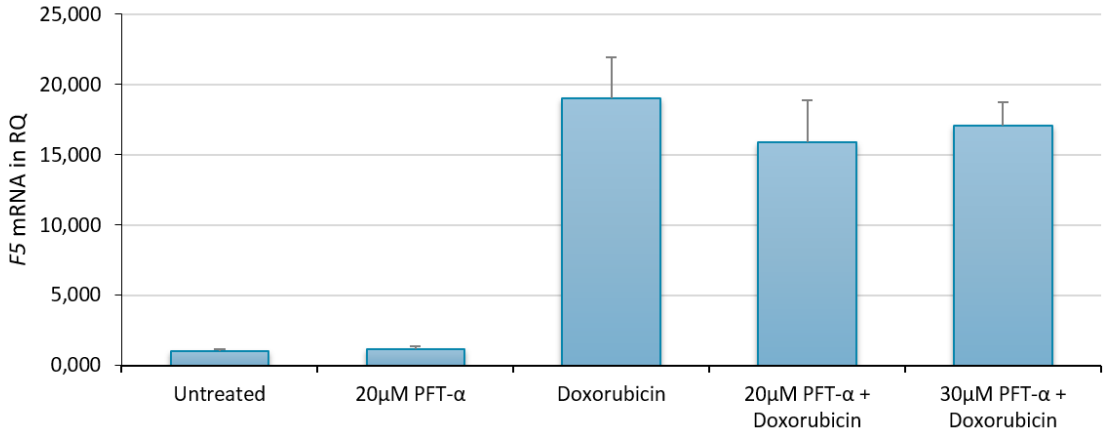

Figure 35. *F5* mRNA expression in SUM102 cells treated with doxorubicin and PFT-α. SUM102 cells were untreated (control) or treated with PFT-α 20 µM, 1 µM doxorubicin, PFT-α 20 µM + 1 µM doxorubicin or PFT-α 30 µM + 1 µM doxorubicin. Cells were pre-treated for 2 hours with PFT-α and 24 hours of doxorubicin, before harvested after 48 hours. *F5* expression was measured using qRT-PCR and normalized agains GAPDH as endogenous conrol. Results are expressed as RQ values compared to control cells Mean values (n=6) + SD from three independent experiments are shown.

In contrast to MDA-MB-231 and MCF-7, adding of two different doses of PFT-α to the SUM102 cells, the induced *F5* mRNA was only slightly reduced by 1,1-1,2-fold compared to cells treated with doxorubicin only (Figure 35). When comparing, the doxorubicin treated cells with cells added 20 µM (p=0,09), or 30 µM (p=0,21) PFT-α, the differences was not significant. This indicates a lower influence of p53 on *F5* mRNA expression in SUM102.

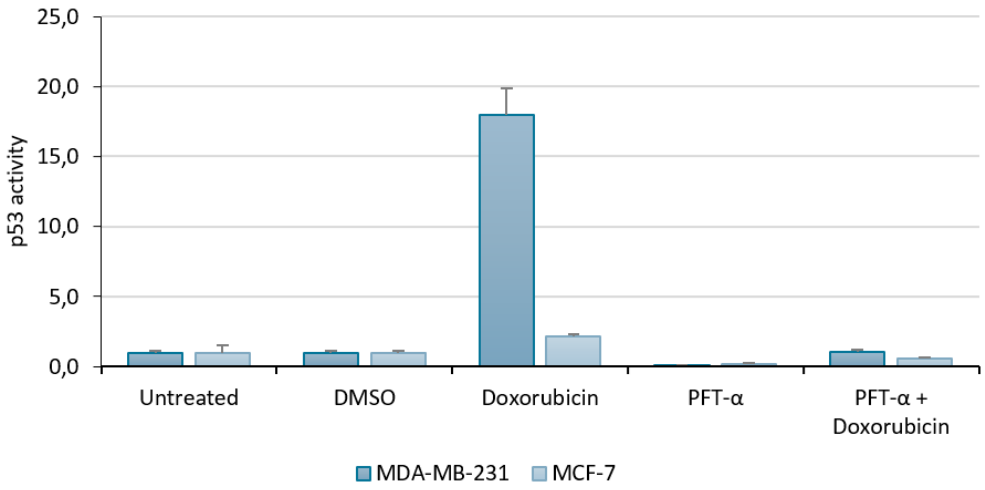

Figure 36. Effect on p53 activity in MDA-MB-231 and MCF-7 cells treated with 1 µM doxorubicin and PFT-α. The p53 activity shown as mean values (n=8) + SD of one experiment.

To verify the p53-inhibitory potential for the p53 inhibitor PFT-α, the activity of p53 was measured with Luciferase reporter assay in MDA-MB-231 and MCF-7 cells. MDA-MB-231 cells showed an 18-fold increase in p53 activity when treated with 1 µM doxorubicin (Figure 36), and when exposed to PFT-α the p53 activity was completely abolished. When adding both 1 µM doxorubicin and PFT-α, the p53 activity was reduced to the same level as the untreated cells (Figure 36). MCF-7 cells exposed to 1 µM doxorubicin showed a 2-fold increase in p53 activity compared to untreated cells. Showing that the p53 activity is less induced in doxorubicin treated MCF-7 compared to MDA-MB-231. The exposure to PFT-α or doxorubicin and PFT-α in combination, confirmed inhibition of p53 activity also in the MCF-7 cells (Figure 36).

4.2.3 Effect of doxorubicin on cell growth in breast cancer cell lines

To study how breast cancer cell lines with different FV expression and p53 mutation status responded to increasing concentrations of doxorubicin on cell growth, the p53 mutant cell lines MDA-MB-231 and MDA-MB-436, and the p53 wild type cells MCF-7 and SUM102, were used. A previous study from our lab showed that SUM102 had highest endogenous expression of FV among the four selected cell lines, while the FV expression was quite similar in the other three cell lines (data not shown).

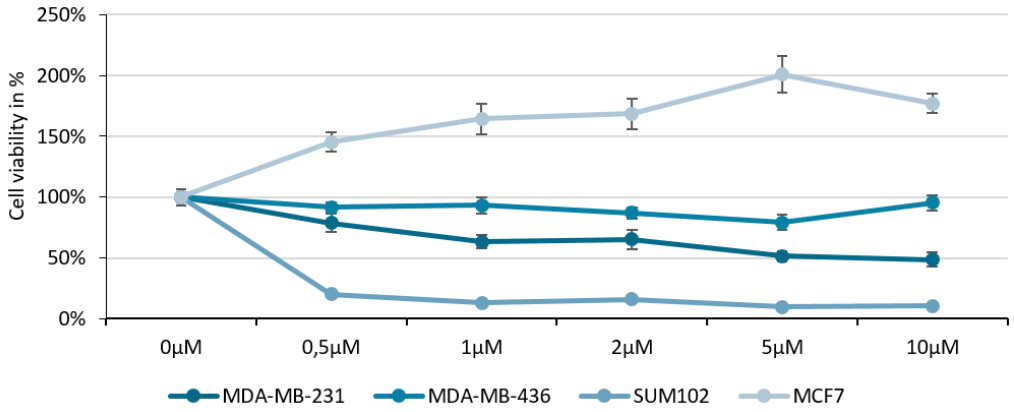

Figure 37. Cell viability measured by Wst-1 for MDA-MB-231, MDA-MB-436, SUM102 andMCF-7 cell lines. Cells were exposed to different concentrations of doxorubicin over a 24 hour period. Concentrations between 0 µM and 10 µM were used and **%** median and SEM of untreated cells are shown (n=15) from three independent experiments.

IC_{50} is a measure of drug sensitivity, which indicates the concentration where 50% of the cells are dead. The two cell lines with p53 mutations were both quite resistant to doxorubicin treatment, however, MDA-MB-231 was more sensitive to doxorubicin compared to MDA-MB-436 (Figure 37). The p53wt cell line SUM102 demonstrated to be most sensitive to doxorubicin, reaching 20% cell viability after only 0,5 µM of doxorubicin and an estimated IC_{50} of 0,3 µM. The other p53wt cell line, MCF-7 was on the other hand the most resistant cell type, which continued to grow under doxorubicin treatment (Figure 37).

The role of FV in doxorubicin induced cell growth

To study the effect of FV on cell viability in presence of doxorubicin, the breast cancer cell lines MDA-MB-231, MCF-7 and SUM102 were treated with FV antibody in addition to doxorubicin.

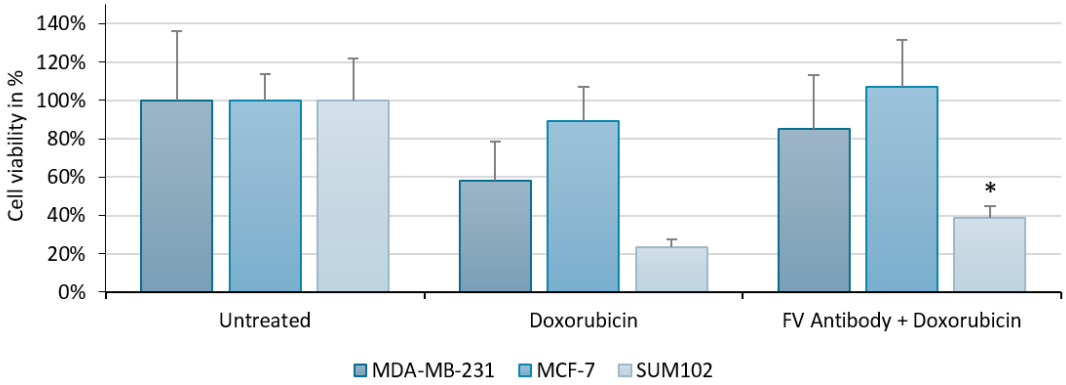

Figure 38. Cell viability measured by Wst-1 in MDA-MB-231, MCF-7 and SUM102 treated with doxorubicin and FV antibody. Viability was measured 24 hours after doxorubicin treatment (1 µM) and compared to control cells. Median from two experiments (n=6) are shown + SEM. Significant diffrence ($p \leq 0,05$) marked with *.

Comparable to Figure 37, cells treated with 1 µM doxorubicin showed a general trend towards reduced cell viability compared to control cells. Interestingly, the cells generally showed a small increase in viability after adding of FV antibody compared to cells treated with doxorubicin alone (Figure 38).

When compared with only doxorubicin treated cells, MDA-MB-231 cells showed a 46% increase in cell viability, when cells were blocked with FV antibody before adding of the same amount of doxorubicin (p=0,7) (Figure 38).

When blocking the MCF-7 cells with FV antibody before adding doxorubicin, the cell viability increased by 20% compared to doxorubicin treatment alone (p=0,6) (Figure 38).

Similarly, in SUM102 cells, blocking with FV antibody resulted in a 70% increased cell viability compared to doxorubicin treatment alone (p=0,03) (Figure 38). These results indicate that FV influence the doxorubicin induced cell death.

5. Discussion

There is increasing evidence that cancer increases the risk of thrombosis (Boccaccio & Comoglio 2009; Timp et al. 2013). In addition, the procoagulant state in cancer has been shown to associate with tumour progression. In fact, the cancer cells themselves are capable of producing procoagulants, thereby directly activating the coagulation cascade. Understanding the underlying molecular mechanisms of cancer and haemostasis associations, could provide more individualized therapy options and better target strategies against cancer and cancer-related thrombosis (Falanga et al. 2003).

The role of coagulation FV in cancer has not yet been studied. However, its role in balancing the coagulation cascade by acting as both a procoagulant and anticoagulant is well known. The main aims of this thesis were to study the effect of FV overexpression in breast cancer cells, and the effect of doxorubicin on *F5* in different breast cancer cells *in vitro*.

5.1 Breast cancer cell lines

Experiments in this thesis were performed using breast cancer cell lines. Cell lines have several advantages as *in vitro* study models, as they are easily acquired, cost-effective and can be frozen and thawed whenever needed. They also possess a longer lifetime due to their infinite potential cell division. It is worth noting that there is also a bias related to the culturing of cells over long periods, as there is a risk of cross-contamination. To reduce this risk, sterile conditions were strictly enforced in all experiments. Cell lines also require specific culture conditions and are highly sensitive to changes in these conditions. Therefore, the recommended culturing medium and the same serum lot were used and cells were incubated 37°C in a 5% CO_2 atmosphere.

5.2 Creation of a FV overexpression construct

Expression constructs are commonly used to selectively express a gene of interest. Gene overexpression is useful in the study of gene functions, and in establishing functional links (Prelich 2012). Using expression constructs, it is possible to genetically manipulate animal models in order to achieve a better understanding of the disease processes (Haruyama et al. 2009). Obtaining the knowledge of the underlying molecular mechanisms behind these processes will make it possible to develop more individualized treatments for human disease.

A new FV overexpression construct was needed to conduct functional studies of FV in breast cancer cells. To create this new FV construct, we amplified *F5* using FV-wt plasmid as a template. Correctly amplifying a DNA fragment the size[17] of *F5* can be problematic, and a custom PCR for long fragments is essential for this process. We used a long-range PCR, with a high-fidelity LongAmp® Taq DNA Polymerase, facilitated to amplify long DNA fragments with a low mutation rate. Although we used long-range PCR, several new mutations occurred in the *F5* gene during amplification. Most frequently, mutations occurred in exon 13, likely because it is an exon with multiple repetitive areas and the largest exon in the *F5* gene. We did not succeed in creating a FV construct without any new mutations present. One of our plasmids contained only two novel mutations one in exon 2 (c.189) and one in exon 13 (c.2694)[18]. These mutations are synonymous according to the Alamut database, so we elected to continue with site-directed mutagenesis to correct the two novel mutations, enabling us to continue our expression studies with this plasmid. Mutagenesis was successful in correcting the mutation in c.189 (exon 2), however, the second new mutation in c.2694 (exon 13) was located in a repetitive area, and site directed mutagenesis failed to correct the mutation. Based on the QuickChange II XL Site-Directed mutagenesis protocol, it was not possible to meet the criteria for a mutagenesis primer, in the area of the second mutation. We also found that the corrected mutation in c.189 first identified as synonymous by Alamut, later showed to be changing a splicing site, according to Human Splicing Finder. Mutation c.2694 was identified as a synonymous mutation in Alamut, and according to Human Splicing Finder it did not change any splicing site, thus assumed to have no influence on the expression of the gene.

[17] The size of the *F5* gene is described in section 3.1, Figure 9.
[18] Position numbers of the mutations retrieved from Alamut, was measured after A, in ATG (ref.seq. NM_000130.4)

We continue to perform expression experiments with the FV construct containing both mutations c.189 and c.2694, as well as the FV construct containing only mutation c.2694. These novel mutations have not yet been reported, and studies of these mutations could enlighten their function in the FV gene. Plasmids were transfected into the MDA-MB-231 breast cancer cells to study possible differences in FV expression on mRNA level and protein level.

5. 3 Overexpression of FV in MDA-MB-231

We chose the triple negative breast cancer cell line MDA-MB-231 as a model in which to study FV overexpression. The cell model was chosen based on the findings by Tinholt *et al.* (Submitted) showing increased *F5* mRNA expression in a subgroup of patients with aggressive tumours. MDA-MB-231 cells were transfected with the FV-wt plasmid (pMT2-FV-wt), previously tested in the research group, as well as the two new FV plasmids.

F5 mRNA expression in the cells transfected with the FV-wt plasmid showed a high *F5* mRNA expression, as could be anticipated from by previous studies in the research group (data not shown). *F5* mRNA expression was 4-fold lower in the plasmid containing the two novel mutations c.189 and c.2694 compared to the efficiency of the FV-wt plasmid, indicating that at least one of the new mutations had a significant effect on the *F5* mRNA expression. After correction of the c.189 mutation, the *F5* mRNA expression increased significantly compared to the plasmid containing both mutations. Hence, the mutation c.189 in exon 2 had a severe effect on the *F5* transcription, which could be due to the change of a splice site as predicted *in silico*. While the c.189 mutation is synonymous, it has previously been shown that synonymous mutations can alter splicing and result in transcriptional deficiency (Zhu et al. 2012). The possible effect of splicing on gene transcription is well-established (Ward & Cooper 2010).

Previous work in our lab has found that cells transfected with the FV-wt plasmid showed a high secretion of FV protein in the cell media (data not shown). As could be expected from the mRNA expression levels, the amount of secreted protein in cells transfected with the new FV plasmid containing both mutations c.189 and c.2694, was considerably low compared to the FV-wt plasmid. Surprisingly, the resulting FV protein levels after the c.189 mutation had been corrected did not correspond with the considerable difference observed at the mRNA expression level before and after correction of the c.189

mutation. This indicates that the mutation in c.2694, located in the B-domain of the protein, has an impact on the translation of FV protein. This means that the mutation in c.189, located in the A1-domain, affects transcription of *F5* but not its translation. This can be explained on the basis of the many mechanisms between the transcription process to the complete protein (Wahl et al. 2009). The main possibilities for the non-corresponding mRNA and protein levels are post-transcriptional mechanisms, mRNA instability and reduced half-life of proteins (Greenbaum et al. 2003). The non-correspondence between the *F5* mRNA expression and the levels of FV protein suggest post transcriptional changes, either due to unknown effects of mutations or due to post-translational changes to the protein.

More knowledge is also arising on the effect of the silent mutations. Silent mutations that change a codon to a rarer codon could result in a slower transcription, possibly leading to a change in protein folding (Pearson 2006). This could explain the high expression of *F5* mRNA and the low levels of FV we observed. Moreover, the location of mutation c.2694 in an acidic region in the B-domain of the protein may influence the binding efficiency of FVac to APC. Unfortunately, we were unable to correct this mutation and its effect on expression was not confirmed in this thesis.

In summary, none of the new FV plasmids showed a higher protein secretion than the FV-wt plasmid. It is possible that observed differences in expression between the two new FV plasmids and the FV-wt plasmid are due to vectors. There are several differences between these vectors: for instance, the pMT2 vector used in the FV-wt plasmid contains the SV40 promotor, while the pcDNA5/FRT vector used in the new FV plasmids contains the CMV promotor. We suggest that further research utilize a new vector that minimizes differences between plasmids. This vector should contain the same benefits as the pcDNA5/FRT vector, and if possible it should be constructed for use with large DNA fragments. Another possibility is to use a different source for the *F5* template for amplification of the gene, such as cDNA obtained from a healthy person.

5.4 Functional effects of FV overexpression in breast cancer cells

Further on, we studied if the novel mutations in the FV plasmids had any functional effects compared to the FV-wt plasmid.

5.4.1 Effect of FV on *IL6* and *IL8* expression in MDA-MB-231 breast cancer cells

Several studies show inflammation reactions to be associated with cancer (Balkwill & Mantovani 2001). Previous findings in the research group have demonstrated that FV expression was correlated with immune response genes in breast cancer patients (Tinholt et al. Submitted), and an increased expression of *IL6* and *IL8* had been observed with FV-wt plasmid in the lab (data not shown). In this thesis, *IL6* and *IL8* mRNA expression was measured in MDA-MB-231 cells transfected with the three different FV expression plasmids. We investigated whether the differences in *F5* mRNA expressed by each FV plasmid resulted in differences in the expression levels of *IL6* and *IL8*.

Effects on *IL6* and *IL8* expression were measured in the transfected MDA-MB-231 cells. We observed only an 58% increase in *IL8* in the pMT2-FV-wt plasmid. The *IL8* expression seems to be overexpressed in most advanced cancers. Increased expression of *IL8* is associated with tumour growth, an advanced stage of cancer, and reduced survival (Merritt et al. 2008). The increase in *IL8* by the FV-wt plasmid seems to indicate that FV has an effect on *IL8* expression, supporting the hypothesis that FV plays a role in cancer progression. The two new FV expression plasmids on the other hand did not affect the expression of either *IL6* or *IL8*.

Our results suggest that the protein level secreted by FV plasmid with mutations c.189 and c.2694 and the FV plasmid with mutation c.2694 were not sufficient to affect the expression of *IL8*. This supports the suggestion that a thresh hold value of FV protein is necessary to induce *IL8* expression in breast cancer cells.

5.4.2 Effect of FV on p53 activity in MDA-MB-231 breast cancer cells

p53 is a tumour suppressor that induces apoptosis in normal cells. p53 has been shown to be mutated in over 50% of human cancers, and is therefore closely linked to the cancer development (Ozaki & Nakagawara 2011). Activation of p53 occurs in the cell as a response to myriad stressors (Hosako et al. 2007). To study if FV overexpression affects p53 activity in breast cancer cells, two different breast cancer cell lines with different p53 status were used: MDA-MB-231 a functional mutant p53 cell type and Mcf-7 a p53wt cell type.

When transfected with pMT2-FV-wt both MDA-MB-231 and MCF-7 cells showed increased p53 activity, indicating that the activation of p53 was induced by FV. Comparable to the effects on *IL8* above, the two new FV expression plasmids did not increase the p53 activity as much as the FV-wt plasmid. This could be explained by the significantly lower amount of FV protein produced by the cells transfected with the new FV plasmids. Even though the FV plasmid with mutation c.2694 showed a small increase in protein levels compared to the FV plasmid with mutations c.189 and c.2694, protein levels were still insufficient to affect p53 activity to the same extent as the FV-wt plasmid.

In summary, neither of two new FV plasmids affected p53 activity to the same extent as the FV-wt plasmid. Hence, the new FV construct did not show any improvement from the previous version utilized in the lab (FV-wt plasmid). New methods will be necessary for creating a FV construct that can be further used *in vitro* studies.

5.5 Doxorubicin's effect on FV in breast cancer cells

In addition to the resistance in some tumours, one of the most serious problems related to treatment with chemotherapeutic agents, is the increased risk of thrombotic complications. Furthermore, anticoagulant therapy in cancer patients is challenging because these patients have an increased risk of major bleeding. The chemotherapeutic drug doxorubicin has been found to have a direct relationship in inducing coagulation in cancer patients (Woodley-Cook et al. 2006). However, little is known about the molecular mechanisms by which doxorubicin triggers a pro-thrombotic state in cancer patients. Thus, it is important to gain improved knowledge of these underlying mechanisms.

In this thesis, we have studied the effect of doxorubicin on the coagulation FV. Being a key component in the coagulation cascade, FV has not yet been studied for any potential effect of doxorubicin treatment. In our experiment four breast cancer cell lines were exposed to increasing doses of doxorubicin, and at different time points. The results showed that the *F5* expression was induced up to 2-5-fold by 1 µM doxorubicin in three of four cell types (SUM102, MCF-7 and MDA-MB-231). Nevertheless, a doxorubicin concentration of 5 µM in these cells, showed no further increase in *F5* mRNA expression. This may be due to the toxicity of doxorubicin at this high dose. Our results provide more detailed knowledge to the field.

Doxorubicin-induced *F5* mRNA expression in breast cancer cells has not been reported before. However, doxorubicin has previously been shown to influence the APC anticoagulant pathway, in both vascular ECs and in plasma retrieved from breast cancer patients (Mukherjee et al. 2010; Woodley-Cook et al. 2006). Woodley-Cook *et al*. (2006) found that in ECs exposed to doxorubicin the haemostatic balance was affected via downregulation of the APC anticoagulant pathway. A later study by Mukherjee *et al.* (2010) supported this mechanism, finding that the anticoagulant APC pathway was inhibited in women with breast cancer who received chemotherapy. FV plays a major role in the APC pathway as an essential cofactor to APC in the inactivation of FVa and FVIIIa (Cramer et al. 2010). Downregulation of APC leads to a decrease in FVa degradation by APC, resulting in increased FVa concentration and increased coagulation. The doxorubicin induced *F5* mRNA expression in our study could lead to an abundance of FV protein.

Several previous studies have been conducted regarding doxorubicin and its effects on the coagulation initiator TF. In contrast to the induction of *F5* mRNA expression in this thesis, monocytes exposed to doxorubicin showed no increase in *TF* mRNA expression (Swystun et al. 2009; Tsunaka et al. 2016; Walsh et al. 1992). Walsh *et al.* (1992) concluded that exposure to doxorubicin likely enhanced the specific activity of the TF already present. This was supported by Swystun *et al.* (2009), who found that targeting ECs with doxorubicin resulted in increased TF activity, while *TF* mRNA levels were slightly downregulated by doxorubicin and TF antigen levels remained unchanged. Similar to the aforementioned studies, we found an approximate 25% reduction in *TF* mRNA after treating MDA-MB-231 breast cancer cells with doxorubicin (data not shown).

Doxorubicin has been found to induce pro-coagulative effect by inducing the expression of TF and phosphatidylserine on the cell surface of EA.hy926 and the T-lymphoblast cell line Molt4 (Tsunaka et al. 2016). This could imply that doxorubicin induced *F5* does not lead to an anti-coagulant effect. Still, this does not exclude other possible effects of FV in cancer. Increased plasma thrombin-antithrombin complexes as well as increased D-dimer levels have been found in breast and lung cancer patients receiving chemotherapy (Weitz et al. 2002). These results support several coagulation factors being induced by doxorubicin treatment, including FV. In this thesis, the doxorubicin induced *F5* expression in breast cancer cells is interesting, however, doxorubicin's effect on *F5* in ECs, which are also directly exposed to doxorubicin during chemotherapeutic treatment, should also be investigated in future work.

We used three p53 expressing cell types in our experiments (SUM102, MCF-7 and MDA-MB-231) as well as one completely lacking p53 expression (MDA-MB-436). Although MDA-MB-231 is a p53 mutant cell, results in this thesis showed an expression of p53 activity. A distinct increase in mRNA expression of *F5* was observed in all the p53 expressing but no increase was observed in the p53 mutant cell line MDA-MB-436. Given that doxorubicin has been shown to induces cell death through the p53 pathway (Sun et al. 2016), it is interesting to see that no *F5* is induced by doxorubicin in the p53 loss-of-function cell MDA-MB-436. This indicates that doxorubicin influences the expression of *F5* in p53 expressing cells only. This strongly implies that the action of p53 is involved in doxorubicin induced *F5* mRNA expression.

It is interesting to note that previous studies have shown increased *TF* mRNA expression in p53 loss of function cells, but not induced *TF* mRNA expression in p53wt cells. Although these experiments were not conducted to the context of chemotherapeutic treatment (Yu et al. 2005). A more recent study by Tsunaka *et al.* (2016), was conducted using EA.hy926 cells (p53wt) and Molt-4 cells (mutant p53). Here they observed no change in *TF* mRNA expression in either cell type when treated with 1 µM of doxorubicin. However, the Molt-4 cell line was used as a mutant p53, which is one of the most misclassified cell types for p53 status, and has been assigned several controversial statuses (Leroy et al. 2014). This could explain the contradictory results in *TF* mRNA expression in mutant p53 cells in the two studies performed with and without chemotherapeutic treatment (Tsunaka et al. 2016; Yu et al. 2005).

5.5.1 p53s role in doxorubicin induced FV expression

Based on the possible correlation between p53 status and doxorubicin induced *F5* expression (discussed above) it was of interest to directly test if p53 was involved in the doxorubicin induced *F5* expression in the breast cancer cells. p53 was therefore inhibited before exposing the cells to doxorubicin.

Our results revealed a general trend in reduced *F5* expression after inhibition of p53 in all cell lines (MDA-MB-231, MCF-7 and SUM102). Inhibition of p53 is generally known to induce cell viability and resistance to chemotherapy (Chen et al. 2002). It is therefore interesting that the doxorubicin induced *F5* is reduced upon p53 inhibition. In MDA-MB-231 and MCF-7 cells, the inhibition of p53 by PFT-α led to a significant reduction in the doxorubicin induced *F5* expression. Since doxorubicin functions by inducing activation of p53, to induce cell apoptosis (Wang et al. 2004), this supports a connection between FV activity and doxorubicin, in doxorubicin apoptotic action.

The doxorubicin sensitive SUM102 differed from the results observed in the two aforementioned cell lines. Doxorubicin induced *F5* mRNA expression was only reduced by 11-16% when p53 was inhibited, and this result was not statistically significant. The observed decrease may be explained by the SUM102 high endogenous FV expression. We cannot rule out the possibility of the decrease being a result of the non-resistant feature of SUM102 to doxorubicin, or due to other potentially unknown mutations in key cancer genes in this cell line. However, these results do imply that p53 activity has a limited influence on doxorubicin-induced *F5* mRNA expression in SUM102.

To confirm the PFT-α inhibitory effect, we measured the activity of p53 in MDA-MB-231 and MCF-7 cells. Cells were treated with both doxorubicin, as well as p53 inhibition by PFT-α before doxorubicin treatment. Interestingly, we observed an 18-fold increase in p53 activity in MDA-MB-231 treated with doxorubicin. These results provide compelling evidence that the mutation in p53 in MDA-MB-231 does not completely inhibit activation of p53 by doxorubicin. Doxorubicin resistance observed in these cells therefore, cannot be explained by lack of p53 function, and the reason for resistance remains unclear. This conclusion is further supported by the successful inhibition of p53 in MDA-MB-231 cells not exposed to doxorubicin, which showed a barely detectable p53 activity after PFT-α inhibition.

The p53-activating function of doxorubicin is also supported by the minor increase of p53 activity we observed when p53 inhibited MDA-MB-231 cells were exposed to doxorubicin.

A similar pattern was observed in MCF-7 cells, but with lower level of p53 activity. The small increase in doxorubicin induced a 2-fold increase in p53 activity, which may help to explain the high resistance to doxorubicin in these cells[19]. MCF-7 is a p53-wt cell type, and sequencing studies performed by our group support this conclusion (data not shown). MCF-7 has been previously shown to be a doxorubicin sensitive cell line (Tsou et al. 2015), which may indicate that a change in the p53 protein after transcription, is affecting its activity. Other mechanisms and effect may be involved and future studies should further investigate the resistance of this p53wt cell line.

5.5.2 Doxorubicin's effect on cell growth in breast cancer cells

The anticancer chemotherapeutic drug doxorubicin functions by interfering with DNA transcription and replication. It acts to stabilize Top2, preventing transcription and generate free radicals that induce cell apoptosis (Tacar et al. 2013). p53 plays an essential role in DNA damage-induced apoptosis and is therefore considered an important contributor in cytotoxicity of antitumour agents (Lowe et al. 1993).

In this thesis, cell viability was investigated in the cell lines SUM102, MCF-7, MDA-MB-231 and MDA-MB-436 treated with increasing concentrations of the doxorubicin. Results showed continuous cell growth in MCF-7 cells[20]. Resistance was evident in both MDA-MB-231 and MDA-MB-436 cell lines, where we observed little or no cell death, and neither of the cells reached IC_{50}. In contrast, we observed a rapid cell death in the very sensitive SUM102 cells, which reached IC_{50} after 0,3 µM doxorubicin treatment.

The doxorubicin resistance in the MCF-7 cells we observed is in accordance with previous observations of no apoptosis in doxorubicin treated MFC-7 cells (Chen et al. 2002). However, Tsou *et al.* (2015) used a different MCF-7/wt (not resistant to doxorubicin) cell line, which showed a high sensitivity to doxorubicin treatment. They also created a doxorubicin resistant MCF-7 cell line and found downregulation of p53wt genes together with upregulation of mutant p53 genes. This suggests the p53 plays an essential role in resistance

[19] Described in chapter 4.2.3
[20] Described in chapter 4.2.3

to doxorubicin in these cells. These results contrast with the findings of the present study, where our p53wt MCF-7 cell line appeared to be doxorubicin resistant. The study from Tsou *et al.* (2015) additionally point out that p53 mutations are frequently found in ER-negative cells. One explanation for these variations in cells from different studies can be the possibility of biological differences in cell lines between different laboratories (Osborne et al. 1987). Another possible explanation might be that doxorubicin affects different pathways, which in turn may mediate apoptosis in various tumour cells (Chen et al. 2002).

Doxorubicin resistance we observed in both MDA-MB-231 and MDA-MB-436, is in agreements with previous findings of resistance to doxorubicin treatment (Aroui et al. 2009; Lefley et al. 2008). The fact that both are mutant p53 cell lines (Huovinen et al. 2011) also supports the findings by Tsou *et al.* (2015), who reported that p53wt cells were sensitive and p53 mutant cells were resistant to doxorubicin, and suggest that p53 mutant cells show a trend in doxorubicin resistance. This is in addition is supported by the same study suggesting p53 mutations most frequently occurs in ER-negative cells (Tsou et al. 2015), in regards to both MDA-MB-231 and MDA-MB-436 being ER-negative. It has been shown that p53 mutations do not directly lead to loss of p53 protein response (Huovinen et al. 2011). In addition MDA-MB-231 has been found to express the p53 protein following a functional mutation, while MDA-MB-436 showed barely detectable expression of the p53 protein (Hollestelle et al. 2010). This could explain the minor differences in cell viability between these two cell lines in our study. Findings in other ER-positive cells expressing resistance to doxorubicin treatment support the observed resistance in our study (Jiang et al. 2013).

In contrast with the other cell lines, SUM102 cells showed a significant sensitivity to doxorubicin exposure in terms of cell viability. Correspondingly, p53wt cells are generally more sensitive to treatment of doxorubicin than p53 mutant cells (Tsou et al. 2015). Although Tsou *et al.* (2015) claims a frequent harbouring of p53 mutations in ER-negative cells, our SUM102 cells are still p53wt cells even though they are ER-negative. ER-negative cells are generally known to be more resistant to chemotherapy than ER-positive cells. Based on the results from this thesis the sensitivity of SUM102 seems to have a connection to its p53 status, shown in the past to be observed in sensitive cells. This is in addition supported by findings in the MCF-7 cell line, where a development from sensitive MCF-7 p53wt cell to a resistant MCF-7 cell, resulted in mutation in p53 (Tsou et al. 2015). Previous work in our group has shown SUM102 to have the highest endogenous expression of FV among the four cell lines used (data not shown), this may indicate that the presence of FV is affecting the

sensitivity of cells to doxorubicin treatment. FV levels may also partly explain the increased variation we observed in SUM102 and MCF-7, where MCF-7 showed 91% lower expression of endogenous FV compared to SUM102. Further investigation is needed to explore this area.

In summary, the results presented here largely correspond with previous research. However, the MCF-7 cell line should be investigated further for potential alteration or encountered mutations. Future investigation of the role of FV expression in doxorubicin treatment may prove fruitful.

FVs role in doxorubicin induced cell death

Based on the knowledge obtained from the cell survival experiment under increasing concentrations of doxorubicin, it was of interest to further investigate the relation between doxorubicin treatment and FV expression. To this end, the three cells expressing p53 protein were inhibited with a FV antibody before exposed to 1 µM of doxorubicin, and cell viability was measured.

All doxorubicin treated cells showed a general, though not significant, increase in viability when FV was inhibited, compared to treatment with doxorubicin only. These results indicate that the FV expression may increase sensitivity to doxorubicin. However, we performed 2 experiments with limited replications, and our work should be repeated to strengthen these findings. The small effect seen, may be due to little protein expressed, and therefore not much protein to be blocked. The high sensitivity of SUM102 to doxorubicin may be partially explained by the higher endogenous FV expression, compared to MCF-7 and MDA-MB-231.

Together, our results suggested that FV contributes to cell sensitivity to doxorubicin treatment. These findings could help to improve cancer treatment. Still, the FV protein levels in the cells used in this thesis were moderate, and it would be valuable to expand on our work and further explore the mechanisms involved, for example by trying a knockdown of FV by siRNA in cancer cells with high endogenous FV expression. Such experiments would further increase our knowledge of FVs role in cancer and doxorubicin treatment.

6. Conclusions

This thesis was performed to gain a better understanding of the role of coagulation FV in breast cancer. An expression study of the effects of overexpression of FV and functional effects, as well as the effect of doxorubicin treatment on FV, was conducted in breast cancer cells *in vitro*. Our results suggest that FV plays a role in the doxorubicin induced apoptosis through the p53 pathway.

The specific conclusions for this thesis are as follows:

I. Expression and functional effects of FV overexpression in MDA-MB-231 *in vitro*:

- The newly constructed FV overexpression plasmid pcDNA5/FRT-FV(c.2694), transfected in MDA-MD-231 breast cancer cells, showed increased expression of *F5* mRNA but low levels and secretion of FV protein in comparison to FV-wt plasmid.
- Overexpression in MDA-MB-231 cells of pMT2-FV-wt showed significant increased expression of inflammatory marker *IL8*. In contrast, the pcDNA5/FRT-FV(c.189/c.2694) and pcDNA5/FRT-FV(c.2694) showed no increase in inflammatory markers.
- Overexpression of pMT2-FV-wt in MDA-MB-231 and MCF-7 cells showed a high increase in p53 protein activity in both cell lines. In comparison, a decrease in p53 activity was seen in both pcDNA5/FRT-FV(c.189/c.2694) and pcDNA5/FRT-FV(c.2694). This indicates that the protein levels in both of the new FV plasmids were too low to affect the activity of p53 at the same level as pMT2-FV-wt.

II. Effect of doxorubicin in breast cancer cell lines:

- Doxorubicin treatment induced *F5* mRNA expression in all p53 expressing cell lines (MDA-MB-231, MCF-7 and SUM102).
- The doxorubicin induced *F5* mRNA expression decreased significantly upon inhibition of p53 in both MDA-MB-231 and MCF-7 cells. The decrease in doxorubicin induced *F5* mRNA after inhibition of p53 in SUM102 was not significant.
- The effect on cell viability of inhibiting FV before doxorubicin treatment showed an increase in viability in all cells compared to only doxorubicin treated cells. The increase was only significant in SUM102 and not significant in MDA-MB-231 and MCF-7.

7. References

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Appendix

Appendix 1.

Table S1. Showing primer sequences for sanger sequencing of the *F5* gene.

Primer name	Primer sequence 5'- 3'
F5-1F	TGGGGGAGCCAAGGGACAGA
F5-2F	CTCGGGGCCAGAATTATTCTCCATTCA
F5-3F	CATCGCCTCTGGGCTAATAGG
F5-4F	AGATTTTTGAACCTCCAGAATCTACAGTCA
F5-5F	GGAGGAAAGAGTAGACTGAAGAAAAGCCA
F5-6F	ATGACTCTCTCTCCAGAACTCAGTCAG
F5-7F	GGAAGAGGTCCAGAGCAGTGAAGA
F5-8F	TGGTTTAAGGAAGATAATGCTGTTCAGCCA
F5-9F	GGCCCCTTCTGCCTGGTTCA
F5-10F	AGAGAATCAGTTTGACCCACCTATTGT
F5-11F	CCCCCAATCATTTCCAGGTTTATCCGT
F5-1R	AGGTGTATTCTCGGCCTGGAGC
F5-2R	ATGCTATAGGGGCGGCTGGC
F5-3R	CCACGCATGGGGAAGAGGGT
F5-4R	AGCCAAATGCCATCTCCCAACCA
F5-5R	AGGATCTGTGACTGGGGTCTGA
F5-6R	TCCGGGAGAAGGGTGGTGTCA
F5-7R	GGATAACATCATCCACTTCAGCTCTGA
F5-8R	ACACTCCAAGCATTATAAGATCCACCA
F5-9R	TCCCTGCTCACTGTAGTGGATGGTAT

Appendix 2.

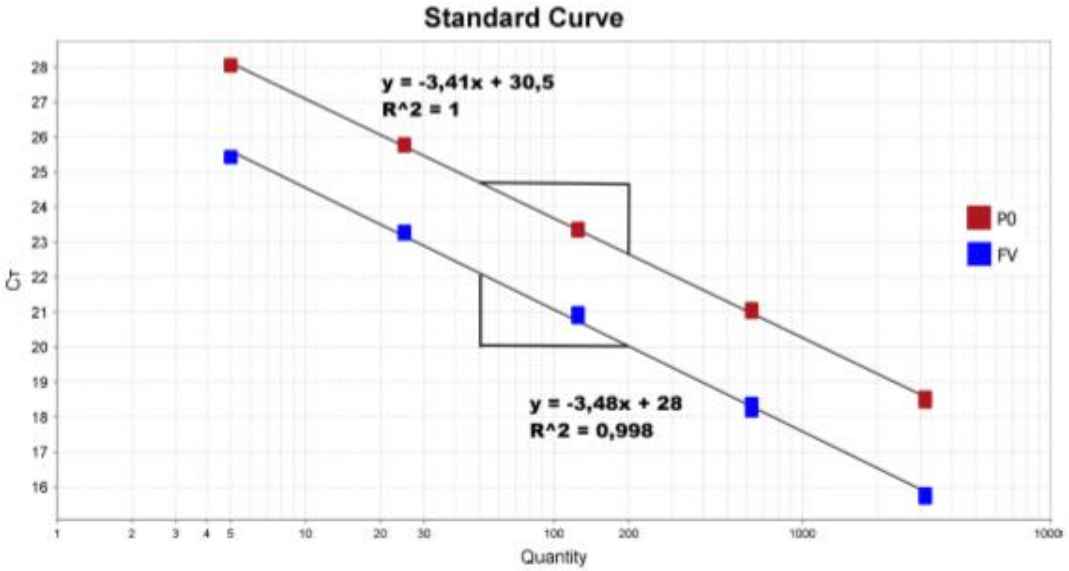

Figure S1. Validation of TaqMan assay performed previously in our research group. Here displaying FV and P0, in this thesis only FV assay wereused. The slop values, R^2 and efficiency rate is here indicated for both assays. FV efficiency was then calculated to 93,8%.

Appendix 3.

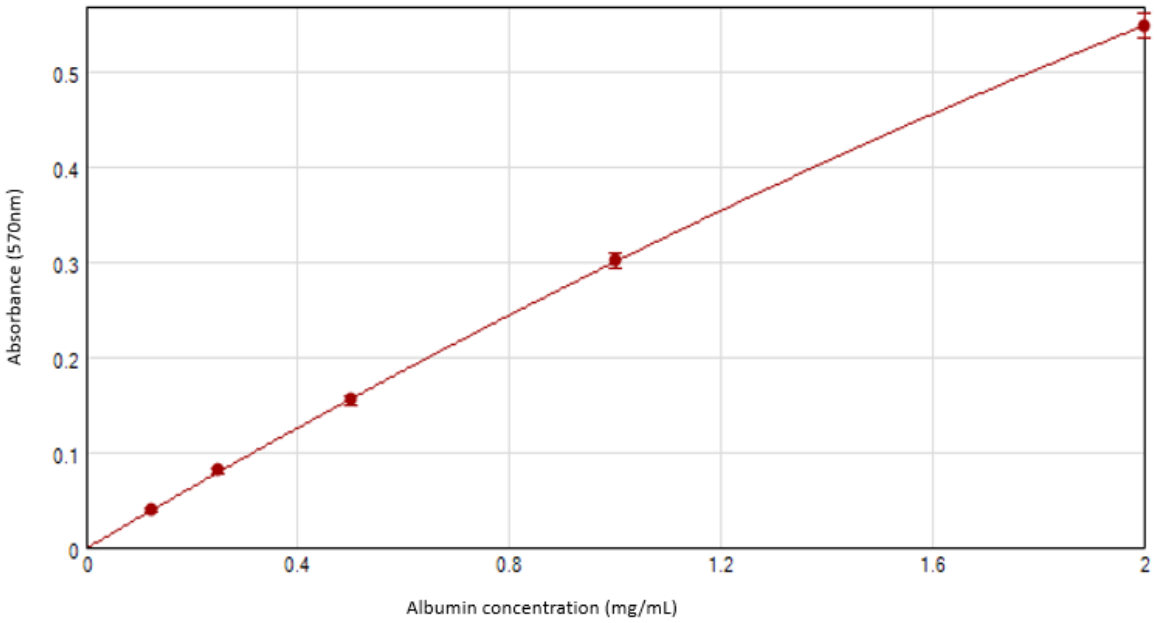

Figure S2. Standard curve displaying albumin concentration at 570nm absorbance. Standard curve used in measuring total protein from RIPA cell lysate. Used in normalization of protein levels when performing ELISA.

Appendix 4.

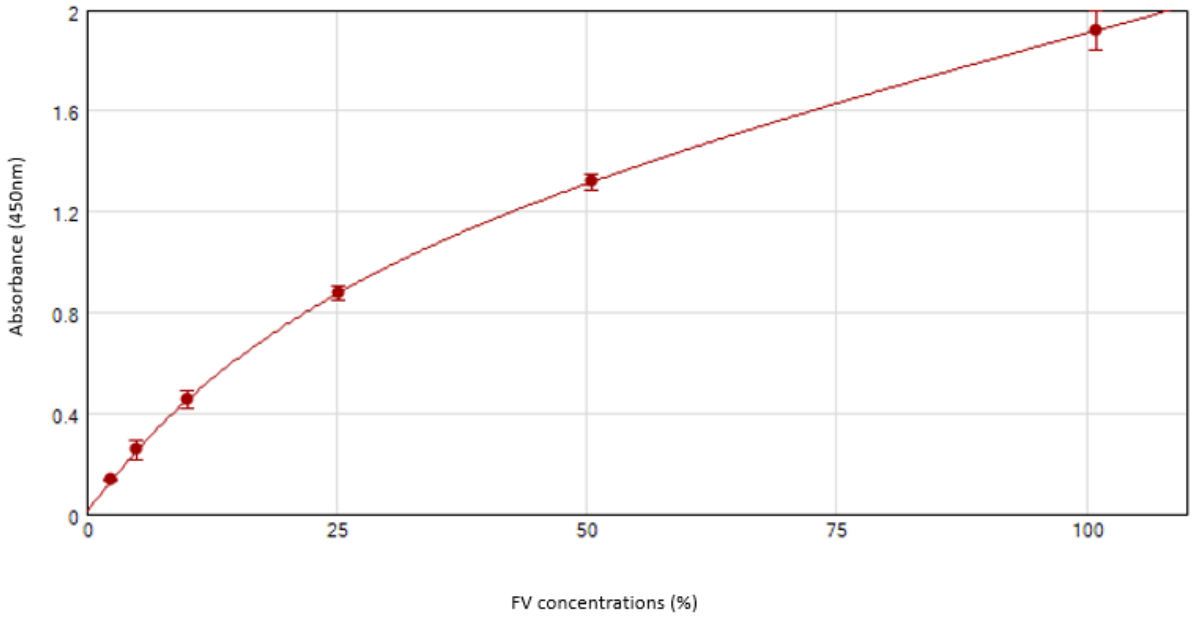

Figure S3. Standard curve displaying FV concentrations in % at 450nm absorbance.
Standard curve used in measuring FV amount in unknown samples from ELISA.

Appendix 5.

Appendix 5. contains complete lists of kits, qRT-PCR assays, reagents and chemicals, antibody/inhibitor, cell types, vectors, instruments and software used in this thesis.

Table S2. Kits and suppliers.

Kits	Supplier	Catalogue number
Agencourt® CleanSEQ®	Beckman Coulter, CA, USA	A2915 4
BigDye® Terminator v3.1 Cycle Sequencing Kit	Thermo Fisher Scientific, Waltham, MA USA	4337455
Cignal Reporter Assay Kit	QIAGEN, Alameda, CA, USA	301005
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific, Waltham, MA USA	4368814
Lipofectamine® 3000 Transfection Reagent	Invitrogen, Carlsbad, CA, USA	L3000008
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, MA USA	23225
RNAqueous® Total RNA Isolation Kit	Thermo Fisher Scientific, Waltham, MA USA	AM1912
ZymoPURE™ Plasmid Maxiprep Kit	Zymo Research, Irvine, CA, USA	D4202 & D4203
Zyppy™ Plasmid Miniprep Kit	Zymo Research, Irvine, CA, USA	D4036, D4019 & D4037

Table S3. TaqMan Assays, supplier and ID.

TaqMan Assays for qRT-PCR	ID	Supplier	Catalogue number
Factor 5 (*F5*)	Hs00914120_m1	Thermo Scientific	4331182
Interleukin 6 (*IL6*)	Hs00174131_m1	Thermo Scientific	4331182
Interleukin 8 (*IL8*)	**Hs00174103_m1**	Thermo Scientific	4331182
Phosphomannomutase 1 (PMM1)	Hs00963625_m1	Thermo Scientific	4351372
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	Hs99999905_m1	Thermo Scientific	4331182

Table S4. Antibody and inhibitor with supplier

Antibody/inhibitor	Supplier	Catalogue number
Anti-Human Factor V	Haematologic Technologies Inc.	AHV-5146
Pifithrin-α	Sigma-Aldrich®	511296-88-1

Table S5. Vectors and suppliers

Vector	Supplier	Catalogue number
pMT2-FV-wt (corrected Version of pMT2-V)	pMT2-V: ATCC	ATCC® 40515™
pcDNA5/FRT	Thermo Fisher Scientific, Waltham, MA USA	V601020

Table S6. Cell type and suppliers

Cell type	Supplier	Catalogue number
OneShot® TOP10 Chemically Component Cells, *Escherichia coli*	Invitrogen, Carlsbad, CA, USA	C4040-03
MCF-7	ATCC, Manassas, USA	ATCC® HTB-22™
MDA-MB-231	ATCC, Manassas, USA	ATCC® HTB-26™
MDA-MB-436	ATCC, Manassas, USA	ATCC® HTB-30™
SUM102	Asterand Bioscience's	CVCL_3421

Table S7. Reagents and chemicals with suppliers

Reagents and chemicals	Supplier	Catalogue number
Ampicillin, Sodium Salt	Calbiochem®	171254
Agar-Agar	Merck, Darmstadt, Germany	101614
Ampicillin, Sodium Salt	Calbiochem®	171254
BlueJuice™ Gel Loading Buffer (10X)	Thermo Fisher Scientific, Waltham, MA USA	10816015
Cell proliferation reagent WST-1	Sigma-Aldrich®	11644807001
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific, Waltham, MA, USA	
Dulbecco's phosphate-buffered saline	Thermo Fisher Scientific, Waltham, MA, USA	14190250
Fetal Bovine Serum	Biowest, nuaillè, France	Si86H-500
GeneRuler 1kb DNA ladder	Fermentas, Vilnius, Lithuania	5M0311
GelStar® nucleic acid gel stain	FMC BioProducts, Rockland,ME USA	50535
Halt™ Protease & Phosphatase Inhibitor Cocktail (x100)	Thermo Fisher Scientific, Waltham, MA USA	1861281
HuMEC Basal Serum-Free Medium	Thermo Fisher Scientific, Waltham, MA USA	12753018
HuMEC Supplement Kit	Thermo Fisher Scientific, Waltham, MA USA	12755013
Lipofectamine® 3000 Transfection Reagent	Thermo Fisher Scientific, Waltham, MA USA	L3000008
Methanol	Emsure®	1.06009.2511
NaOH	Merck, Darmstadt, Germany	1064981000
OptiTMEM Reduced Serum Medium	Thermo Fisher Scientific, Waltham, MA USA	31985T062

Roswell Park Memorial Institute (RPMI) 1640	Thermo Fisher Scientific, Waltham, MA USA	11875093
Reagent A 100	Chemo Metec A/S	910T0003
Reagent B	Chemo Metec A/S	910T0002
RIPA buffer	Thermo Fisher Scientific, Waltham, MA USA	R0278
S.O.C. medium (Super Optimal Broth with Catabolite Repression)	Invitrogen™	1749148
Sodium Chloride (NaCl)	Merck, Darmstadt, Germany	1.06404.5000
Trypsin EDTA	Lonza, Verviers, Belgium	BE17T161E
Tryptone	Merck, Darmstadt, Germany	119311000
UltraPure™ 10x TAE buffer	Thermo Fisher Scientific, Waltham, MA USA	15558-042
Yeast Extract	Sigma-Aldrich Norway AS Oslo, Norway	Y1625T250G

Table S8. Instruments and suppliers.

Instruments	Supplier
ABI 3730 DNA Analyzer	*Applied Biosystems®*
ASYS atlantis 4	Applied Biosystems®
BioMek FX	Beckman Coulter
DELFIA® plate shake	Wallac
Forma™ 370 Steril-Cycle™ CO_2 Incubator	Thermo Scientific
ImageQuant™ LAS 4000 imagine system	GE Healthcare
Incubator	Termaks A/S
NanoDrop® ND-1000	Nikon
Nikon eclipse TE300	Nikon
Nikon eclipse Ts2-FL	Nikon
NucleoCounter® NC-100™	ChemoMetec A/S

Table S9. Software and suppliers.

Software	Supplier
Alamut Visual	Interactive Biosoftware
DNA sequencing analysing software v5.1	Applied Biosystems®
Human Splicing Finder	Genetics and Bioinformatics Team
Snap Gene	GSL Biotech LLC

Appendix 6.

Appendix 6. contains lists of solutions used in this thesis.

Table S10. Lurian Broth (LB) medium.

Step 1	Step 2	Step 3
10g Tryptone	Adding MQ-H_2O up to 1L	Adjust pH to 7 with 12M HCl before autoclaved. Adding of 100µg/mL ampicillin before use.
5g Yeast extract		
10g NaCl		

Table S11. LB-agar plates

Step 1	Step 2	Step 3
10g Tryptone	Adding MQ-H_2O up to 1L	Adjust pH to 7 with 12M HCl before autoclaved and 100µg/mL ampicillin added before spread on plates.
5g Yeast extract		
10g NaCl		
15g agar		

RIPA lysis buffer with inhibitors:

- 1 x RIPA buffer
- 1:100 Protease and phosphatase Inhibitor cocktail (100x)

1% Agarose gel:

- 1 x Agarose agar
- 1:10 TAE buffer (10x)

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