The mechanisms of cellular signaling by Factor VII activating protease (FSAP)

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II Abbreviations

AKT	Protein kinase B
ANGII	Angiotensin II
APC	Activated protein C
AREG	Amphiregulin
AT	Anti thrombin
bFGF	Basic fibroblast growth factor
CCL2	Chemokine (C-C motif) ligand 2
CCR2	C-C chemokine receptor type 2
CRE	cAMP responsive element
CREB	CRE binding protein
DAMPs	Damaged associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
EC	Endothelial cells
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EPCR	Endothelial cell protein C receptor
EGF	Epidermal Growth Factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial Nitric Oxide Synthase
EREG	Epiregulin
FSAP	Factor VII activating protease
GPCR	G protein-coupled receptor
HABP2	Hyaluronic acid binding protein-2
HRP	Horse radish peroxidase
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MCL	Myosin light chain
MI-FSAP	Marburg I FSAP (G534E)
MMP	Matrix metalloproteiase
NE	Neutrophil Elastase
NET	Neutrophil extracellular traps
NO	Nitric oxide
NTR	N terminal region of FSAP
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-Activated receptors
PDGF-BB	Platelet-derived growth factor-BB
PI3K	phosphatidylinositol 3-kinase
PKC	Protein Kinase C
PLG	Plasminogen
PMA	Phorbol 12-myristate 13-acetate
PPACK	D-Phenylalanyl-prolyl-arginyl Chloromethyl Ketone
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophorese

SELE	E-selectin
SERPIN	Serine protease inhibitors
SNP	Single nucleotide polymorphism
SPD	Serine protease domain
TF	Tissue Factor
TFPI	Tissue Factor Plasminogen inhibitor
TGFβ	Transforming growth factor β
TM	Thrombomodulin
tPA	Tissue Plasminogen activator
uPA	Urokinase Plasminogen activator
VCAM1	Vascular adhesion molecule 1
VSMC	Vascular smooth muscle cells
vWF	Von Willebrand Factor
WT	Wild type

III List of papers included

Paper I **Factor VII activating protease (FSAP) regulates the expression of inflammatory genes in vascular smooth muscle and endothelial cells.**

Kristina Byskov, Thomas Boettger, Paul F. Ruehle, Nis Valentin Nielsen, Michael Etscheid, Sandip M. Kanse

Atherosclerosis, Aug 2017, In Press.
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Paper II Manuscript
Protease Activated Receptor-2 (PAR-2) serves as a dominant receptor for Factor VII Activating Protease (FSAP).
Kristina Byskov, Sylvain Le Gall, Bernd Thiede, Eric Camerer, Sandip M. Kanse

1 Introduction

1.1 The vascular system

1.1.1 Blood

The multifaceted tasks of blood are carried out by different components. The erythrocytes are the main cell type of the blood whose function is to transport oxygen. They do not have a nucleus and lack most cellular organelles, and their cytoplasm consists mainly of hemoglobin. Platelets are also anuclear and are important in the process of haemostasis. Activation of platelets leads to platelet adhesion, aggregation and the formation of a blood clot. Platelets release a broad variety of growth factors, e.g. platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). The last group of cells in the blood is the leukocytes, consisting of neutrophils, basophils, eosinophils, monocytes and lymphocytes. Leukocytes are immune cells, and each subtype has different functions. The acellular fluid in blood is called plasma. Plasma mostly consists of electrolytes, nutrients, lipoproteins and proteins. Amongst others these are coagulation factors, immunoglobulins, carrier proteins and growth regulatory factors.

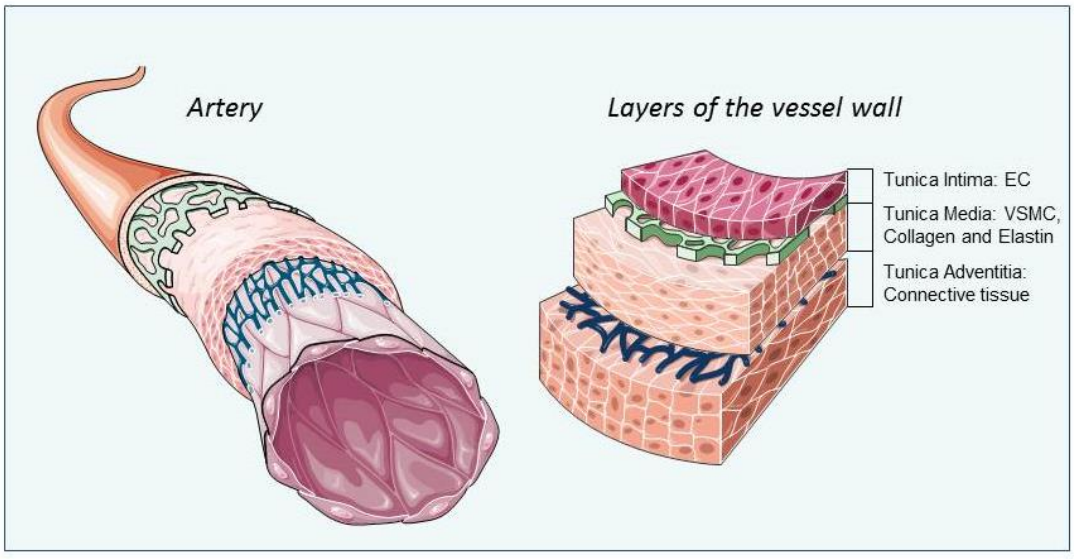

Figure 1: Structural organization of an artery. The three main layers (tunica Intima, tunica Media and tunica Adventitia) are indicated with its components.

Blood vessels are subdivided into arteries and veins that are connected by capillaries. The main difference between arteries and veins is that arteries have a

thicker medial layer than veins that allows them to cope with the higher pressure. Blood vessels are composed of three main layers, tunica intima, tunica medial and tunica adventitia. The inner layer, tunica intima, consists primarily of endothelial cells (EC) and basal lamina. The medial layer is composed primarily of vascular smooth muscle cells (VSMC), elastic fibers and collagen fibers and the outer tunica adventitia layer is composed fibroblasts, nerve endings and the vasa vasorum[1].

1.1.2 Cells of the vessel wall

Endothelial cells (EC): EC line the vessel wall and thereby serve as a barrier to prevent blood from escaping the vascular system. EC serve as important regulators of both physiological and pathological functions e.g. modulating vascular tone, regulation of inflammation, controlling haemostasis as well as mediating signal transduction to the intima layer of the vessel by expression and secretion of regulatory factors, cytokines and mitogens.

The endothelium regulates vascular tone by secretion of relaxing factors such as nitric oxide (NO) and prostaglandin I_2 and contracting factors such as endothelin I, angiotensin II and thromboxane A_2[1] and thereby signal to VSMC to either contract or relax in order to regulate blood pressure. NO not only regulates vascular tone, it also inhibits EC activation through decrease of adhesion molecules like vascular cell adhesion molecule 1 (VCAM1) and E-selectin (SELE) as well as lowering interleukin (IL) -6 and IL-8 release[2]. Together with sequestering P-selectin in Weibel-Palade bodies, NO protects the endothelial layer from leukocyte attachment as well as platelet activation[3]. Thus, NO is one of the key mediators produced by EC.

Endothelial dysfunction is characterized by increased vascular constriction, endothelial permeability, leukocyte recruitment and the failure to limit coagulation. Upon inflammatory activation of EC, endothelial permeability is increased through myosin light chain (MLC) activation or through reorganization of the cytoskeleton and intracellular adhesion molecules such as VE-cadherin. Leukocytes adhere to EC through increased release of selectins and adhesion molecules, a feature which initiates leukocyte rolling and trans-endothelial migration[3]. Furthermore, activation of EC changes the endothelial layer from being anti-coagulant expressing e.g. tissue factor pathway inhibitor (TFPI) and thrombomodulin (TM)[3] to become pro-coagulant.

Binding of thrombin to TM activates protein C (PC), which is a key inhibitor of coagulation[4]. Endothelial activation changes the balance between tissue factor (TF) and TFPI in favor of TF.

Angiogenesis, the growth of new blood vessels, occurs under physiological condition throughout life, but also under certain pathological conditions e.g. during cancer growth and metastasis, where formation of new blood supplies is necessary for the tumors to survive and continue to grow. Under appropriate stimuli, EC proliferate and migrate into tissue creating a new plexus of capillaries and vessels. The main inducers of angiogenesis are vascular endothelial growth factor (VEGF) A, fibroblast growth factor (FGF) 2 and angiopoietin-1 and -2.

VSMC: The medial layers of the vessel wall consist of VSMC together with extra cellular matrix (ECM) components produced by the VSMC. The main function of VSMC is to regulate vascular tone through vasoconstriction and vasodilation. In vascular injury, hypertension or aging, various factors trigger a change from the contractile to the synthetic phenotype, causing increased ECM remodeling and fibrosis[5]. Regulators of VSMC plasticity include PDGF-BB[6, 7] and transforming growth factor-β (TGF-β)[8, 9]. PDGF-BB promotes a synthetic phenotype[10] and TGFβ favors a contractile phenotype[8]. The main intracellular regulators for both phenotypes are transcription factors and serum response factor. Activation of serum response factor promotes the contractile phenotype and its suppression promotes the synthetic phenotype[5, 11].

Upon vascular injury, as in e.g. angioplasty or stent insertion, excessive VSMC proliferation results in accumulation of VSMC in the intimal layer, called the neointima or restenosis. Vascular injury triggers apoptosis of VSMC, cell proliferation, migration and secretion of mitogens and chemotactic factors, e.g. IL-6 which all promote neointima formation[12]. Apart from PDGF other factors, such as thrombin, are also involved[13]. VSMC within neointima lose the ability to contract[14] and combined with ECM proteins secreted by the VSMC lead to vascular stiffness and lumen narrowing. In the early phase of neointima formation, monocytes in the intima secrete cytokines and chemokines which activate VSMC proliferation and migration. VSMC migrate from the medial layer and accumulate on the surface of the plaque creating a fibrous cap covering the core of the plaque. VSMC inside the plaque differentiate into

macrophage-like cells[15, 16] thereby facilitating continued growth and inflammation in the plaque core[17]. VSMC can also become phagocytic which allows the uptake of lipoproteins, thereby converting them into foam cells. Macrophage-like VSMC can also phagocyte apoptotic VSMC[18] in the atherosclerotic plaque which in turn triggers a further secretion of chemokines[19].

Leukocytes: Leukocytes can be divided into five main categories; basophils, eosinophils, neutrophils, lymphocytes and monocyte/macrophages. Monocytes are circulating phagocytic cells which, upon stimulation such as inflammatory cytokines, migrate into tissue and differentiate into macrophages. Macrophages can also be derived from other fetal precursor cells within the tissue[20]. Macrophages can participate in both progression of inflammation and in its resolution and are further subdivided into MI which are pro-inflammatory and M2 which have anti-inflammatory properties[21].

Adhesion and migration of monocytes into the intimal layer, is a hallmark of atherosclerosis progression[22]. Through phagocytosis, macrophages ingest dead cells, lipids and cell debris converting them into lipid-rich foam cells. In addition, monocyte-derived macrophages secrete pro-inflammatory cytokines and growth factors[20, 22]. The primary factor for recruiting monocytes to the sub-endothelium is monocyte chemoattractant protein-1 (MCP-1) also called chemokine (C-C motif) ligand-2 (CCL2)[23] but other factors have also been shown to mediate leukocyte recruitment such as IL-8/IL-8 receptor CXCR-2[24] and protease activated receptors (PARs) [25, 26]. Activation of monocytes in the blood causes expression of TF which regulates coagulation and secretes pro-inflammatory cytokines and is pro-thrombotic[27].

Neutrophils are the phagocytic granulated immune cells, which serve as the immediate effector cell against microbial infections. Activation of neutrophils is often the first response to acute infection but neutrophils can also be activated by sterile inflammation or tissue damage. After activation, neutrophils migrate to the site of infection through rolling, adhesion, crawling and endothelial transmigration. Neutrophils kill pathogens by one of three mechanisms; phagocytosis, granular secretion or neutrophil extracellular traps (NETs) formation, also called NETosis. The role of neutrophils in acute inflammation is mainly protective but can also be pro-inflammatory[27,28].

Platelets: Platelets play an important role in haemostasis and thrombosis. Platelets express a number of key adhesion molecules and receptors that enable them to perform their functions. The platelet receptors which mediate adhesion are primarily glycoproteins and integrins e.g., GP IIb/IIIa binds fibrinogen, GP VI and α2β1 binds collagen, GP Ib/IX/V binds Von Willebrand factor (vWF)[29]. Initially, platelets adhere to subendothelial matrix through receptor/ ligand binding. Firm adhesion, which leads to signal transduction in the platelet, and incorporation of new platelets into the clot, follows later. Circulating platelets are attracted and activated by secretion of granules containing soluble agonists from the already activated platelets in the clot. Examples of agonists are adenosine diphosphate (ADP) and thrombin. Three types of granules in platelets exist; dense granules secreting ions, amines and nucleotides, alpha granules containing adhesion molecules and proteins and lysosomal granules contain enzymes and proteases that are all involved in the haemostasis process[29].

1.2 Diseases involving the vessel wall

1.2.1 Atherosclerosis and vascular remodeling.

Atherosclerosis is an inflammatory disease resulting in plaque formation in arteries thereby narrowing the vascular lumen. The atherosclerotic plaque consist of a necrotic core containing lipid filled cells and a fibrous cap containing VSMC and ECM proteins[17]. In addition to lumen narrowing, rupture of the atherosclerotic plaque can cause platelet aggregation and thrombus formation, which leads to an acute cessation of blood flow[22]. The major pathological consequences of atherosclerosis are myocardial infarction or stroke which are the leading causes of death world wide[30].

Endothelial dysfunction promotes monocyte adhesion and migration[31] and is considered to be the initiating event in atherosclerosis. Monocytes undergo transition into macrophages which engulf lipids and become foam cells which form the lipid core[22]. These cells secrete inflammatory cytokines which stimulate VSMC to undergo phenotypic transition into the synthetic phenotype and gain the ability to proliferate

and migrate in to the atherosclerotic lesion. VSMC either stabilize the plaque by creating the fibrous cap or increase the plaque mass. Plaques become vulnerable when the cap becomes thin and the necrotic core expands through e.g. apoptosis of VSMC. Apoptosis lowers the number of VSMC as well as increasing intimal inflammation[32]. VSMC can undergo phenotypic transition into macrophage-like cells and thereby become foam cells, a process regulated by the transcription factor KLF4[33]. When an atherosclerotic plaque ruptures, coagulation factors in the blood come into contact with TF and ECM proteins expressed by cells in the lipid core. This triggers platelet aggregation and thrombus formation, which consequently can cause myocardial infarction or stroke[22].

1.2.2 Thrombosis.

Haemostasis is the physiological ability to form blood clots that is necessary to avoid bleeding after injury. Pathological clot formation in the absence of injury is called thrombosis. Depending on its location, excessive thrombus formation can lead to myocardial infarction, pulmonary embolism, stroke and venous thrombosis. Thrombus formation is initiated when the endothelial layer is damaged and platelets come into contact with and adhere to subendothelial collagen or vWF. Exposed TF activates the coagulation cascade, which ultimately leads to thrombin generation[34]. Thrombin in turn converts fibrinogen to fibrin which stabilizes and cross-links the fibrin clot and activates platelets[35-38]. High shear stress or epinephrine can also induce thrombosis via endothelial vWF secretion. Platelet glycoproteins bind the vWF fibers which activate the platelets causing thrombin secretion and platelet aggregation[34]. Furthermore, leukocytes can also induce thrombus formation through expression of TF, release of cytokines, granular proteases like neutrophil elastase (NE) and cathepsin G, and damaged associated molecular patterns (DAMPs) like histones, nucleic acids and NETs[27].

In addition to the pro-coagulant functions, thrombin also activates PC via binding to TM and thereby generating activated PC (APC). APC has anti-coagulant activity through proteolytic cleavage and inhibition of FVa and FVIIIa[4]. Other anti-coagulants are antithrombin (AT) and TFPI. Genetic modulation of one of these

factors as well as their modulators and co-factors is a risk factor for the development of thrombosis[4, 34].

Arterial thrombosis and venous thrombosis differ in the mechanism by which they are activated and propagated. Arterial thrombosis is mediated by atherosclerotic plaque rupture and is characterized by platelet aggregation leading to the formation of a platelet-rich thrombus. Venous thrombosis is most often initiated on intact EC which become activated by altered shear stress or inflammation and thereby allows binding of platelets and leukocytes. Activated platelets and leukocytes express TF which in turn activates coagulation. Venous thrombi are normally fibrin-rich and easily detach from the endothelial surface and travel to a distal site causing e.g. pulmonary embolism[34]. These differences arise mainly due to different sheer stress and the composition of the vessel wall and also reflect the different treatment regimens.

The primary effector of clot dissolution, called fibrinolysis, is plasmin which degrades fibrin. Plasmin is generated from the inactive zymogen plasminogen (PLG) via proteolytic cleavage by urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA). Generation of plasmin from PLG is enhanced when tPA bind to either fibrin or annexin 2 on EC forming a complex with PLG or when uPA binds to uPAR or integrins on leukocytes[39]. Leukocytes promote fibrinolysis through expression of uPA and its receptor uPAR and through plasminogen receptors[27]. Serine protease inhibitors (SERPINs) tightly regulate the process of fibrinolysis. These are the plasmin inhibitor α2-antiplasmin, protease nexin or the plasminogen activator inhibitor (PAI-1) which inhibits both tPA and uPA. Lastly, thrombin activated fibrinolysis inhibitor attenuates fibrinolysis through blocking binding sites for tPA and PLG on fibrin and annexin 2[39].

1.3 Factor VII activating protease (FSAP)

FSAP is a serine-protease secreted by hepatocytes into the circulation in its inactive zymogen form[40]. Structurally, FSAP contains three epidermal growth factor (EGF) domains, a kringle domain and a serine protease domain[41] (Figure 2). Upon activation it is converted into a two-chain form bridged by a disulfide bond between the heavy and light chain[42]. It was originally identified as hyaluronic acid binding protein-2, hence the name of the gene is *HABP2*[41]. Later the protease was

characterized for its proteolytic actions on Factor VII and was therefore given the name FSAP[43]. FSAP is primarily expressed and secreted into the circulation by hepatocytes but is also expressed in monocytes and monocyte-derived macrophages[44] as well as by different lung cancer cell lines[45]. Studies on FSAP$^{-/-}$ mice and the linkage of single nucleotide polymorphisms (SNP) with diseases suggest that FSAP is a multifunctional protease with diverse pathophysiological functions.

1.3.1 Activation, Function and biochemical properties

Activation: The zymogen form (pro-FSAP) is activated by polyanions such as DNA, RNA and heparin[46] and also by apoptotic and necrotic cells[47], nucleosomes[48] and histones[49]. Histones can activate both, purified and plasma pro-FSAP[49], whereas polyanion activation of FSAP is limited to purified pro-FSAP only. RNA or heparin binding and activation of pro-FSAP is mediated by a positively charged cluster in the EGF3 domain of FSAP[46]. Histones bind to the negatively charged N-terminal region (NTR) of FSAP[49]. Based on these observations an auto-activation model has been proposed whereby pro-FSAP molecules, in the presence of a charged polymer, interact with one another and undergo binding and conformational change. This initiates the initial cleavage events[42].

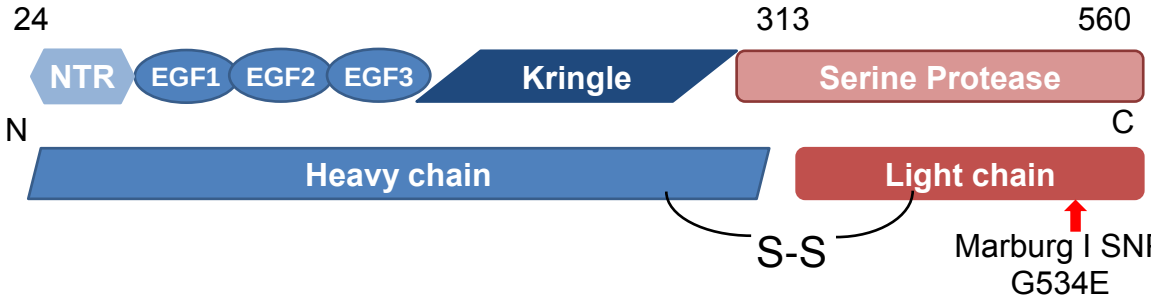

Figure 2. Schematic representation of FSAP in its single-chain zymogen form (top) and two-chain active form (bottom). The localization of the MI-SNP (G534E) is indicated.

Functions of FSAP: Although, originally defined on the basis of FVII activation[43], FVII was found to be a poor substrate for FSAP[50]. Other substrates for FSAP include; pro-uPA[51, 52], fibrinogen and fibronectin[52], TFPI[53], PDGF-BB[54], bFGF/EGF[55] and kininogen[49, 56]. In addition FSAP degrades histones from necrotic

cells, which may be related to decreasing the toxic effects of histones[57]. FSAP substrates are defined by a cluster of basic amino acids on the non-prime site and an arginine or lysine in position P1[58].

Once FSAP is activated *in vivo*, it can be rapidly inactivated by various inhibitors such as α1-proteinase inhibitor, antithrombin III, CI inhibitor, α2-plasmin inhibitor[51, 59-61], PAI-1[62], protease nexin-1[63] and TFPI[64].

1.3.2 FSAP in health and disease

Marburg I polymorphism: Two naturally occurring SNPs have been found in the FSAP encoding gene; G534E, also called Marburg I (MI) and E393Q, also called Marburg II (MII)[65] polymorphism. The MI-SNP results in lower proteolytic activity whereas MII-SNP showed no changes in activity compared with wild type FSAP[50, 65, 66]. While no diseases have been linked with the MII-SNP, increasing attention has been drawn to the linkage between the MI-SNP and various diseases. Recently, modelling the structure of the serine protease domain (SPD) of WT-FSAP and MI-FSAP, suggested that the one amino acid change resulted in conformational change which blocked activation of pro-MI-FSAP[67].

In patients with carotid stenosis, the presence of the MI-SNP was associated with a worse outcome[68]. In a large correlation study of stroke patients, MI was found to increase the risk for stroke and mortality[69] and increased FSAP activity and antigen levels were found in ischemic stroke patients[70]. Furthermore low FSAP antigen level was associated with recanalization in stroke patients after tPA treatment[71]. FSAP activity was also found to be increased in acute coronary syndrome patients[72] and in patients with deep venous thrombosis[73]. Taken together, these studies show that FSAP is activated in various cardiovascular diseases and it appears that low proteolytic activity of FSAP is related to increased disease risk.

Phenotype of FSAP$^{-/-}$ mice: In a mouse model of ischemic stroke, FSAP$^{-/-}$ mice showed increased stroke volume and worsened neurological deficit compared to wild type (WT) animals[74]. This was related to an increase in leukocyte infiltration and apoptosis after stroke. In a mouse model of vascular injury, a larger neo-intima

formation in FSAP$^{-/-}$ mice was associated with an increased leukocyte infiltration and VSMC proliferation[75]. FSAP, but not the proteolytically inactive MI-FSAP or chemically inhibited-FSAP, protected the vessel wall against neo-intima formation. A mechanism based on proteolytic cleavage of PDGF-BB was suggested although not directly shown *in vivo*[66]. FSAP$^{-/-}$ animals showed delay in thrombus formation and had increased re-bleeding[76]. The effect of FSAP on thrombosis was suggested to be due to proteolytic cleavage of TFPI, a known substrate for FSAP[53] and one of the main regulators of coagulation[4]. Bile duct ligation on FSAP$^{-/-}$ mice showed increased liver fibrosis and activation of hepatic stellate cells compared to wild type animals[77] supporting a protective role of FSAP in inflammation-related conditions.

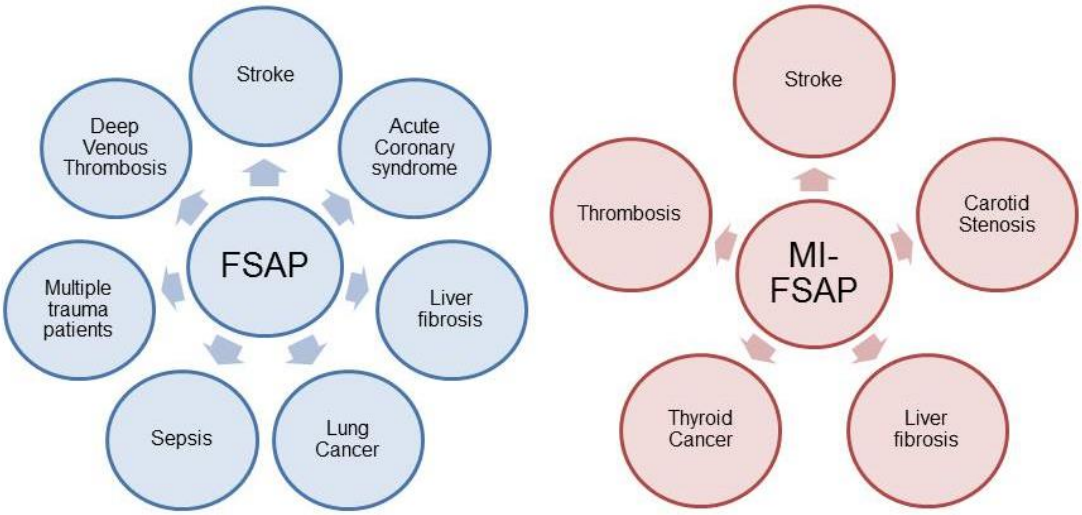

Figure 3. Conditions associated with altered circulating FSAP levels (left) and those associated with an altered risk in carriers of MI-SNP (right).

Role of FSAP In cancer: Mirzapoiazova and co-workers found that different lung carcinoma cell lines as well as tissue from non-small lung carcinomas expressed FSAP. Cell migration, endothelial barrier function and tumor growth was impaired if endogenous FSAP expression was blocked by shRNA[45]. The MI-SNP has also been associated with familial nonmedullary thyroid cancer[78] however this finding has been questioned by various researchers[79]. It was shown that MI- but not WT-FSAP over-expression increased colony formation and migration in various cell lines and vice versa when inhibiting expression of FSAP using siRNA[78]. In addition, MI-FSAP but

not WT-FSAP could transform mouse fibroblasts and thereby allow anchorage independent growth in soft agar[78].

Role of FSAP in inflammation: There is increasing evidence for a link between FSAP and inflammation. FSAP expression is increased in sepsis, meningococcal sepsis, post-operative acute-phase patients[47] and liver fibrosis with low grade inflammation[77]. In a mouse model of chronic liver injury, FSAP expression was increased at an early stage but decreased later[80].

FSAP is activated by apoptotic and necrotic cells[47] and mediates the release of nucleosomes from late apoptotic cells[48]. In systemic lupus erythematous FSAP is not able to mediate nucleosome release due to autoantibody cross-linking to the nucleosomes[81]. In multiple trauma patients FSAP activity was correlated with increased generation of C5a and increased circulating nucleosomes. *In vitro* studies showed that FSAP, but not MI-FSAP, was able to activate both C3 and C5 to generate the anaphylatoxins C3a and C5a[82]. In summary, studies in FSAP knockout mice show a generalized increase in infiltrating leukocytes which indicates that one function of endogenous FSAP is to suppress inflammation[74, 75, 77].

1.3.3 Cellular effects of FSAP

The mechanism of action of FSAP on a cellular level is still poorly understood. One proposed model for FSAP mediated cell activation is through cleavage and inhibition or activation of growth factors. In VSMC, FSAP inhibits PDGF-BB-mediated mitogen activated protein kinase (MAPK) activation, cell proliferation and migration through proteolytic cleavage[54, 66]. Similarly, inhibition of VEGF by FSAP has also been proposed[83]. On the other hand conversion of the zymogen pro-BMP-2 to active BMP-2 increased its activity towards osteoblastic transformation of cells[84]. Another proposed mechanism of cellular activation is through proteolytic cleavage of bFGF. FSAP caused a decrease in apoptosis in EC[55] and blocking the FGFR1 led to inhibition of FSAP induced MAPK signaling and proliferation in fibroblasts[85]. Combined these studies suggest an activating role of FSAP on bFGF/FGFR1

signaling. Additional FSAP was also shown to cleave kininogen and thereby release bradykinin, suggesting yet another cellular activation mechanism[56].

FSAP can also activate cells in a growth factor-independent manner[74, 86]. In astrocytes and neurons, FSAP seems to mediate a cyto-protective response via PAR1 and the phosphatidylinositol 3-kinase (PI3K)/ Protein kinase 3 (AKT) pathway[74]. It was shown to regulate barrier function through a PAR1 and PAR3 dependent and Rho/Rho-associated protein kinase (ROCK) signaling dependent mechanism in EC[86]. FSAP induced a pro-inflammatory response through the transcription factor NFkB in monocytes as well as increasing monocyte adhesion and trans-endothelial migration[87]. These studies together open the possibility of a more direct mechanism of cellular activation by FSAP.

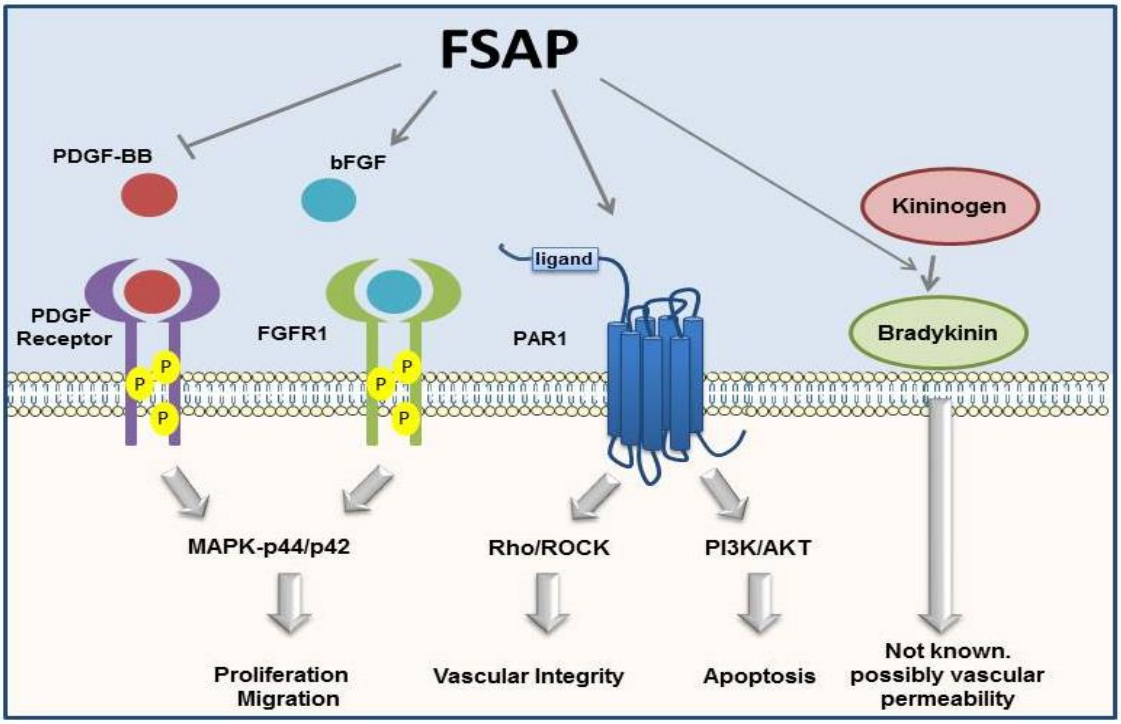

Figure 4. Summary of known cellular functions of FSAP 1) proteolytic inhibition of PDGF-BB 2) proteolytic activation of bFGF, 3) PAR1 mediated signal transduction and 4) proteolytic activation and release of bradykinin.

1.4 Protease Activated Receptors

PARs are a family of four G-protein coupled receptors (GPCR) comprising of PAR1, 2, 3 and 4. PAR signaling is initiated by proteolytic cleavage of the N-terminal end of the receptor which generates a tethered ligand for the receptor. PAR receptor signaling is involved in various pathological conditions e.g. cancer, neurological conditions, inflammatory responses, coagulation and repair mechanisms[88]. PAR1 was the first PAR to be discovered, and was cloned as the platelet receptor for thrombin[35]. PAR1 is primarily expressed in platelets, EC and VSMC, but also in other cells. PAR2 is highly expressed in prostate, small intestine, colon, heart, liver, kidney, pancreas and arteries. In addition PAR2 is expressed in various cancer cell lines[89, 90], EC and VSMC[91]. PAR3 is expressed in heart, liver, kidney, pancreas, thymus, small intestine, colon, stomach, lymphatic node, trachea and bone marrow[36], EC[92] but not in VSMC[91]. PAR4 is expressed in most tissues besides heart, brain, spinal cord, and peripheral blood leukocytes[93] and is expressed in human coronary artery[90]. PARs are activated by a variety of proteases and have an important role in many pathophysiological processes.

1.4.1 Activation and signaling

Canonical cleavage: The classical activation of PARs is through canonical cleavage which is defined by the first identified ligand and proteolytic cleavage for each PAR. PAR1[35], 3[36] and 4[93] were all identified as thrombin receptors; hence thrombin determines the canonical cleavage site for these receptors. Thrombin cleavage generates new N-terminal tethered ligands; S_{42}FLLRN for PAR1[35], T_{39}FRGA for PAR3[36], G_{48}YPGQV for PAR4[93]. PAR2 was identified as a trypsin activated receptor and trypsin therefor determines the canonical cleavage site for PAR2 exposing the S_{37}LIGKV tethered ligand[89, 94]. Agonist peptides, with similar sequence as the tethered ligand, are able to induce a cellular response similar to cleavage by proteases at that particular site and are called activating peptides. In addition to the first identified cleavage sites of the PARs multiple proteases have been shown to activate all four receptors at their canonical cleavage site e.g. FXa and plasmin on PAR1, FXa, FVIIa and tryptase on PAR2. All proteases which cleave PARs are listed together with the cleavage site in Table 1.

Table 1. Ligands and cleavage sites for PAR1-4.

Receptor	Protease	Human Cleavage site	ref
PAR1	Thrombin	R_{41}-SFLLRN	Vu [35]
	FXa	R_{41}-SFLLRN	Staveneitier[95]
	Plasmin	R_{41}-SFLLRN	Kuliopulos[96]
	APC	R_{46}-NPNDKY	Mosnier[97]+ Riewald[98]
	NE	L_{45}-RNPDK…	Mihara[99]
	Proteinase 3	A_{36}-TLDPRSF	Mihara[99]
	MMP-1	D_{39}-PRSFLLRN	Boire[100], Trivedi[101]
	MMP13	S_{42}-FLLRN	Molino
	Cathepsin G	N.D	Sambrano[102]
PAR2	Trypsin	R_{36}-SLIGKV	Bohm[89], Nystedt[94], Nystedt[103]
	TF/FVIIa	R_{36}-SLIGKV	Camerer[104]
	FXa (TF/FVIIa/FXa)	R_{36}-SLIGKV	Camerer[104]
	Tryptase	R_{36}-SLIGKV	Molino[105]
	Matriptase	N.D.	Le Gall[106]
	NE	S_{67}-VLTGK	Ramachandran[107] + Dulon[108]
	Proteinase 3	V_{60}-DEFS	Ramachandran[107]
	Cathepsin G	F_{64}-SASV	Ramachandran[107]+ Dulon[108]
	(Thrombin)	R_{36}-SLIGKV	Mihara[109]
	Cathepsin S	E_{56}-TVFS	Elmirah[110] + Zhao[111]
	KLK	N.D.	
PAR3	Thrombin	K_{38}-TFRGA	Ishihara[36]
	APC	R_{41}-GAPP	Mosnier[97]
	FXa	R_{41}-GAPP	Staveneutier[95]
PAR4	Thrombin	R_{47}-GYPGQV	Xu[93]
	Trypsin	R_{47}-GYPGQV	Gomides[112]
	Plasmin	R_{47}-GYPGQV	Quinton[113]
	Cathepsin G	R_{47}-GYPGQV	Sambrano[102]
	MASP1	N.D.	

Non-canonical cleavage: PARs can also be activated through non-canonical cleavage, which in turn exposes a different tethered ligand. The new tethered ligand causes a different down-stream signal, which is called biased signaling. One example of non-canonical cleavage causing biased signaling is the cleavage of PAR1 at R_{46} by APC[97] which generates the neo-epitope N_{47}PNDKY. This non-canonical cleavage of PAR1 mediates a cytoprotective and anti-inflammatory response vs. cytotoxic and

pro-inflammatory response mediated after cleavage at R_{41}, the canonical thrombin cleavage site[97]. Other examples are neutrophil elastase (NE) which cleave at non-canonical cleavage sites in both PAR1[99] and PAR2[107], matrix metalloproteinase (MMP)1 which cleaves PAR1 at non-canonical D_{39}[101] and Cathepsin S which cleaves at non-canonical site E_{56} of PAR2[111], all creating biased signaling. Furthermore, FXa canonical cleavage at PAR1 but non-canonical cleavage of PAR3 was shown to have, both, thrombin-like and APC-like cellular effects[95].

Disarming: PARs can also be inactivated through disarming of the receptor. By proteolytic cleavage, proteases disarm PARs by cleaving downstream of canonical or non-canonical cleavage sites, thereby preventing other proteases from activating the receptor. MMP1 cleave PAR1 such that canonical thrombin cleavage and activation is prevented[114]. Also NE, cathepsin G and proteinase 3 are known to disarm PAR2[107].

*Cross talk between PARs: P*ARs can form homo- or hetro-dimers which allows cleavage of one PAR to activate another receptor through its tethered ligand. It was shown that the PAR1 activating peptide SFLLR-NH_2[115, 116] or thrombin generated tethered ligand of PAR1 was able to trans-activate PAR2 in EC[117]. Furthermore PAR1-PAR2 trans-activation was shown to change signaling from PKCα to PKCε signaling in EC[118]. Unlike the other three PARs, the tethered ligand of PAR3 is not able to activate PAR3 itself but rather trans-activate the other PARs[36].

Dimerization of PARs also allows for other regulatory effects. PAR1 cleavage at the canonical site leads to signaling and internalization independently of β-arrestin[119]. However, in complex with PAR2, PAR1 signaling and internalization is changed to β-arrestin-mediated endosomal signaling rather than fast lysosomal degradation[120]. Another way of modulating PAR signaling is through G-protein selectivity. PAR1/PAR1 vs. PAR1/PAR3 dimerization was shown to change G-protein preferences; whereas PAR1/PAR1 can bind both $G\alpha_q$ and $G\alpha_{13}$, PAR1/PAR3 dimers prefer $G\alpha_{13}$ binding only[121].

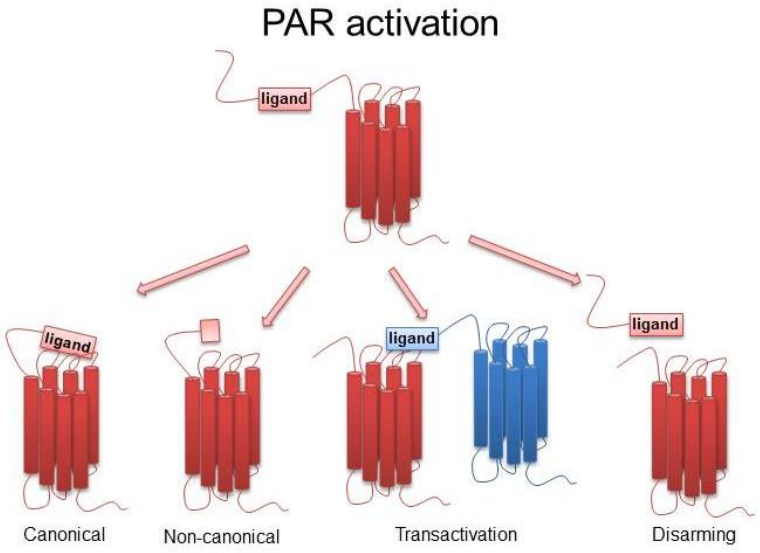

Figure 5. Different activation and inactivation mechanisms of PARs. Cleavage at the N-terminal extracellular tail of PARs can result in 1) canonical cleavage, 2) non-canonical cleavage, 3) transactivation of other receptors or 4) disarming and thereby inactivation of the receptor.

Co-receptors and modulators of PARs: Co-receptors are also involved in PAR activation. APC and FXa require binding to the endothelial proteinase C receptor (EPCR) to facilitate PAR1 or PAR3 cleavage and signaling[98, 122]. FVIIa uses TF as a co-factor for PAR2 cleavage and FXa binds a complex of EPCR, TF/FVIIa to mediate PAR1 and PAR2 cleavage[123]. In addition, the ternary TF/FVIIa/FXa complex is suggested to use matriptase to activate or amplify activation of PAR2[106]. Thus co-receptors concentrate and position the protease in close proximity of PAR and promote more efficient cleavage and activation.

Other PAR regulatory mechanisms: Since PARs are irreversibly cleaved upon activation, desensitization and recycling of the receptors is necessary. After cleavage, the fate of the cleaved PARs is regulated differently depending on the type of receptor as well as the type of activation. Canonical cleavage of PAR1 leads to internalization and lysosomal degradation vary rapidly through a mechanism involving deubiquitylation, dynamin and clathrin-dependent processes[119], whereas canonical cleavage of PAR2 leads to internalization by a slower mechanism[124]. Upon cleavage of PAR2 by trypsin, PAR2 is internalized through β-arrestin binding and transported to early endosomes by Rab5a. From here it is further transported to lysosomes for degradation[125, 126]. Cleavage of PAR2 by NE on the other hand signals

through a mechanism independent of β-arrestin and is not internalized for degradation[107]. In addition APC-activated PAR1 at the R_{46} is dependent of β-arrestin and endosomal trafficking[97], also contributing to the biased signaling of PAR1. Taken together, binding to β-arrestin, internalization to endosomes, lysosomes or staying at the cell membrane after activation, modulates the cellular response.

Coupling to different G-proteins is another mechanism whereby PARs can alter signaling. E.g., trypsin activation of PAR2 is linked to $G\alpha_q$ whereas cathepsin S showed biased signal by coupling to $G\alpha_s$[111]. The same ligand can mediate different signals through linkage to different G-proteins[127]. The knowledge on how PARs are activated is constantly being extended, and draws a more and more complex network of events which all participate in generating a ligand-, cell type- and context-specific signalling.

1.4.2 Role of PARs in vascular pathophysiology

Platelet activation and thrombosis: In humans, PAR1, 3 and 4 were identified as receptors for thrombin[35, 36, 93] whereas PAR3 was shown to serve as the main thrombin receptor on mouse platelets[128]. PAR1 and PAR4 in humans[37] and PAR3 and PAR4 in rodents are important for platelet responses in thrombosis and haemostasis. It was also shown that MMP1 mediates collagen induced platelet aggregation through PAR1 cleavage and thereby causes thrombus formation[101].

Vasoconstriction/vasodilation: PAR1 and PAR2 are important regulators of vascular tone. Activation of PAR1 or PAR2 in mice caused a decrease in blood pressure which was abolished in $PAR1^{-/-}$ and $PAR2^{-/-}$ mice respectively[90, 129, 130]. Activation of both PAR1 and PAR2 increases release of NO which is vasodilatory[131] and blocking NO in the various mouse models abolished the effect of PAR1 and PAR2 activation on vasodilation.

Inflammation: PARs are key regulators of inflammation. $PAR2^{-/-}$ mice showed a delay in leukocyte rolling, and activation of PAR2 decreased leukocyte rolling and adhesion[132]. $PAR2^{-/-}$ mice also showed decreased arthritis[133] and PAR2 was shown

to mediate an inflammatory response by release of neutrophil proteases, such as PR3, leading to IL-18 secretion[134]. Trypsin was shown to induce an inflammatory response through both PAR1 and PAR2[135]. PAR1$^{-/-}$ mice showed decreased lung fibrosis and PAR1 co-localized with macrophages in lung tissue from patients with lung fibrosis[136].

PARs are also involved in sepsis progression. MMP1 cleavage of PAR1 promotes sepsis[137] and inhibition of thrombin signaling together with PAR2 deficiency improved survival and lowered inflammation[138] suggesting that PAR1 as well as PAR2 are involved in the progression of sepsis. On the other hand APC was shown to protect against sepsis and this protective effect was lost by knocking out PAR1[139]. Using agonist and antagonist pepducins against PAR1 Kaneider *et al.* showed a barrier disruptive role of PAR1 in early sepsis whereas activation of PAR1 at late stage sepsis was beneficial[140]. The change in the role of PAR1 in vascular damage is controlled by a change from Rho signaling to Ras signaling and cross-linking with PAR2[140].

Cardio-protective effects: APC has a cytoprotective effect in various pathologies and tissues, and this cytoprotective effect is mediated through cleavage of PAR1 at position R_{46}[97, 141]. Indeed it was shown that APC protects the heart from ischemia reperfusion (I/R) injury through PAR1 signaling. In the early inflammatory phase, APC decreases apoptosis, secretion of the pro-inflammatory cytokine IL-6 and leukocyte infiltration[142]. Activation of PAR2 also protects the heart from I/R injury by decreasing oxidative injury and increased expression and secretion of the anti-inflammatory cytokine TNFα[143]. The positive effect of PAR2 activation on I/R injury was dependent on MAPK and PKC signaling although PI3K/AKT was not involved[144]. Inhibition of PAR1 by antagonists like vorapaxar is protective against myocardial infarction[145].

1.4.3 PARs in VSMC and EC

VSMC: Thrombin stimulates proliferation and migration of VSMC[146, 147] leading to neointima formation[5]. FXa was shown to activate DNA synthesis and activate

MAPK-p44/p42 in a PAR2 dependent manner[148] and pepducin-induced PAR1 activation caused mitogenesis and proliferation that was PAR2 dependent[149]. However, thrombin-stimulated proteoglycan synthesis and smad2 activation was mediated through PAR1 transactivation of TGFBR1[150]. Kallikrein trans-activate the epidermal growth factor receptor (EGFR) through a mechanism involving cleavage of PAR1 and PAR2 which in turn activates ADAM17 to release amphiregulin (AREG) and thereby activate its receptor, EGFR[151]. Thrombin induces IL-6 secretion and mRNA expression in a mechanism involving MAPK and EGFR signaling, and the cAMP responsive element (CRE) transcription factor binding sites in the *IL-6* promotor is necessary for *IL-6* gene expression[147]. Using second generation RNA sequencing, Kamato et al show how 50% of all gene expression induced by thrombin was dependent on transactivation of either EGFR or the TGFBR1. *IL-6*, prostaglandin-endoperoxide synthase 2 (*PTGS2*), *AREG* and *NR4A1* belonged to the genes which were highly upregulated by transactivation[152].

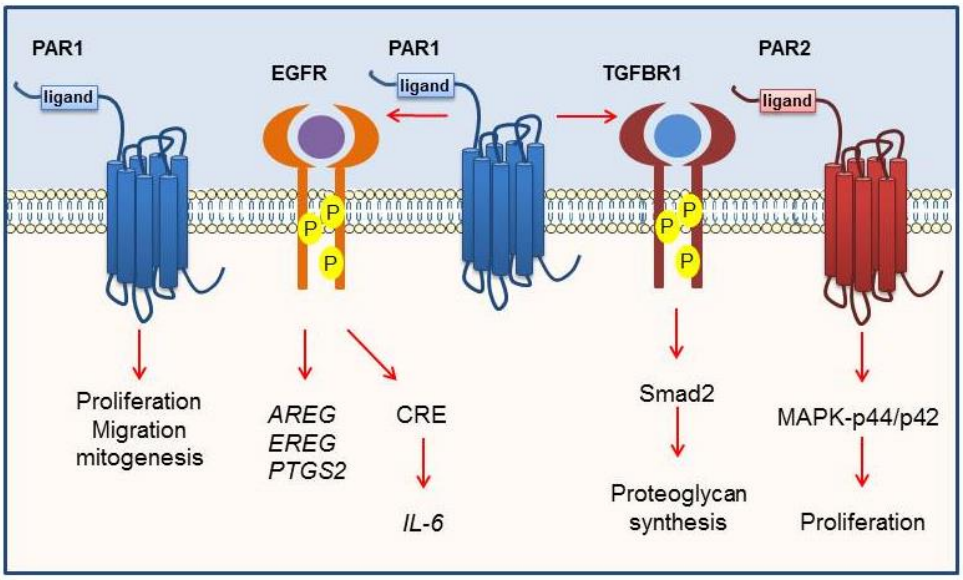

Figure 6. Summary of some major PAR mediated cell signaling events in VSMC as described in the text.

EC: PAR1 mediates vaso-relaxation, however when endothelium is damaged PAR1-mediated response shifts to vaso-constriction[153]. Stimulation of PAR1 and PAR2 showed activation of endothelial NO synthetase (eNOS)[154] in a differential manner. Thrombin-induced PAR1 signal was suggested to trans-activate PAR2[117]. MMP1 was shown to cause endothelial barrier disruption and Rho-GTP activation through

PAR1[137]. APC mediates endothelial barrier protection through PAR1[155] and FXa can mediate endothelial barrier protection through either PAR1 or PAR2[156], and if both PAR1 and PAR2 expression was deleted, no FXa signaling was observed[157]. Activation of PAR1 increases endothelial monolayer permeability and release of vWF through Rho activation and cdc42-GTPase[158].

1.4.4 Use of PARs as targets in disease:

PARs are candidates as drug targets e.g. PAR1 for thrombosis and cardiovascular diseases and PAR2 in inflammatory diseases. To date only the PAR1 antagonist vorapaxar has been approved for secondary prevention after myocardial infarction[159]. Vorapaxar binds to PAR1 in an irreversible manner[160]. One of the major problems with classical PAR antagonists is that they inhibit also the positive effects of PARs e.g., the cytoprotective effects induced by APC.

A new class of antagonists for PAR1, parmodulins targets the cytoplasmic side of PARs and thereby do not interfere with ligand binding. In contrast to classical antagonists, parmodulins can be developed with selectivity for specific effects by blocking specific G protein coupling. Parmodulin-2 showed comparable inhibitory effects towards platelet aggregation, but did not inhibit APC mediated cyto-protective effect on EC, as seen with vorapaxar. In addition, vorapaxar alone causes endothelial dysfunction, and this was not observed with parmodulins[161]. The way parmodulins regulate PAR1 signaling is through altering selectivity for $G\alpha_q$ and not $G\alpha_{12/13.}$

Another type of cell permeable antagonists for GPCR are pepducins. They interact with the effector of GPCRs and thereby antagonize specific cellular effects. The PAR1 pepducin, PZ-128 was successful in phase I clinical trial and is currently in phase II clinical trial[162]. The advantage for the usage of pepducins and parmodulins is that they can be designed in a way so the only antagonize certain cellular effects and remains beneficial effects of PARs intact. To date, no PAR2 antagonists have shown high enough potencies to be clinical relevant; however the antagonist GB88 has shown promise in *in vivo* studies as well as different pepducins, all of which shows partial signaling selective antagonism[119].

Other potentials for the usage of PARs as drug targets are through development of PAR agonist ligands. E.g., a recombinant version of APC (3K3A-APC) is currently being tested in clinical trial for ischemic stroke. 3K3A-APC does not have anti-coagulant activity like WT-APC, but retains its neuroprotective PAR signaling properties[163].

2 Aims of study

Genetic epidemiological studies and experiments with $FSAP^{-/-}$ mice indicate that FSAP is an important mediator in vascular biology. In order to advance our understanding about the pathophysiology of FSAP, more information about its actions on vascular cells is needed.

The overall aim of the current work was;

To study gene expression patterns in FSAP stimulated VSMC and EC and the signaling pathway behind these effects.

To systematically study the role of PARs in mediating the cellular action of FSAP.

3 Summary of results

Paper I: Factor VII activating protease (FSAP) regulates the expression of inflammatory genes in vascular smooth muscle and endothelial cells.

Kristina Byskov, Thomas Boettger, Paul F. Ruehle, Nis Valentin Nielsen, Michael Etscheid, Sandip M. Kanse

In press, Atherosclerosis, Aug 2017, doi.org/10.1016/j.atherosclerosis.2017.08.029

In paper I we characterized the gene expression pattern in FSAP-stimulated VSMC and EC using microarray analysis and functional annotation analysis. The highly upregulated genes; *AREG, IL6* and *PTGS2* in VSMC and *IL8, VCAM1* and *SELE* in EC indicate a stimulaton of a proliferative and especially inflammatory response. Down-regulated genes in VSMC belonged to the group of aldo/keto reductases that metabolise a variety of endogenous and exogenous substrates and included *AKR1B10, AKR1C2, AKR1C3.* Down-regulated genes in EC belonged to the catagories; mitosis, cell division and cytoskeleton organization and included the genes *BRCA1, KIF11, BUB1* and *CENPF* to mention some. We verified the gene expression of the up-regulated genes using qPCR and we were able to confirm the secretion of IL-6 from VSMC and IL-8 from EC using ELISA.

The necessity of the proteolytic activity of FSAP for the regulation of both gene expression and cytokine secretion was confirmed. For this we applied two different serine protease inhibitors, as well as recombinant SPDs proteins of both WT and MI-FSAP. MI-SPD has a very low proteolytic activity and therefore serves as a negative control for WT-FSAP. In VSMC, gene expression was impaired by the PAR1 antagonist vorapaxar but not the PAR2 antagonist ENMD-1068. In EC PAR1 antagonists had no effect on gene expression indicating that PAR1 is not involved in FSAP mediated gene expression in EC. In VSMC the MAPK-p44/p42 (ERK1/2) and cAMP pathways were involved in modulating the changes in gene expression by FSAP.

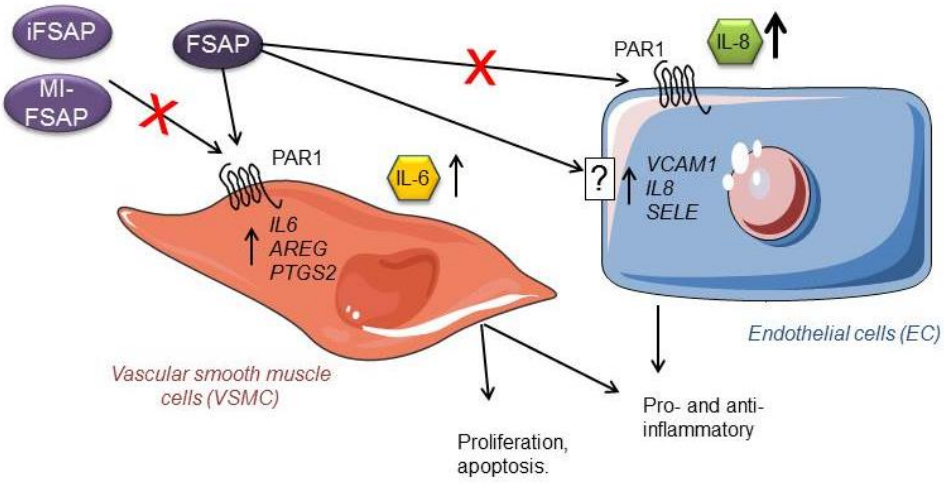

Figure 7. Graphical summary of the main findings in paper. 1) FSAP upregulated inflammation, proliferation and apoptosis-related genes in vascular cells. 2) Proteolytically inactive and Marburg I isoform of FSAP had no effect on cells. 3) Some, but not all effect of FSAP are mediated through PAR1.

Paper II: Protease-Activated Receptor 2 (PAR2) serves as a dominant receptor for Factor VII Activating Protease (FSAP).

Kristina Byskov, Sylvain Le Gall, Bernd Thiede, Eric Camerer, Sandip M. Kanse

(Manuscript in preparation)

In paper II we investigated the role of PARs in mediating FSAP signaling in more detail. We show that PAR2 serves as the dominant receptor for FSAP. This was demonstrated by transfecting HEK293T and A549 cells with PAR constructs harboring an N-terminal secreted alkaline phosphatase (SEAP) tag, which upon proteolytic cleavage is released into the supernatant. PAR1 was also cleaved by FSAP and MAPK p44/p42 cell signaling was stimulated through, both, PAR1 and PAR2 in these cells. PAR3 and PAR4 did not show significant increase in either receptor cleavage or MAPK p44/p42 cell signaling. The requirement for proteolytic active FSAP to mediate cleavage of PAR2 was shown by pre-treating FSAP with the serine protease inhibitor aprotinin and by comparing the activity of WT-SPD and the inactive MI-SPD. Neither FSAP pre-treated with aprotinin nor MI-SPD mediated PAR cleavage or signaling.

Point mutations were introduced in the N-terminal part of PAR2 at potential cleavage sites. K34 and R36 were identified as being important for FSAP-mediated cleavage and cell signaling. Site directed mutagenesis confirmed that R41 was the cleavage site in PAR1. FSAP also cleaved synthetic N-terminal PAR1 and PAR2 peptides at the canonical cleavage sites as determined by mass spectrometry.

Thus, through over-expression of a reporter PAR constructs we could show activation of PAR2 as well as PAR1 in cells by FSAP. The relative contribution of these receptors is dependent on the cell type and alludes to additional regulatory mechanisms.

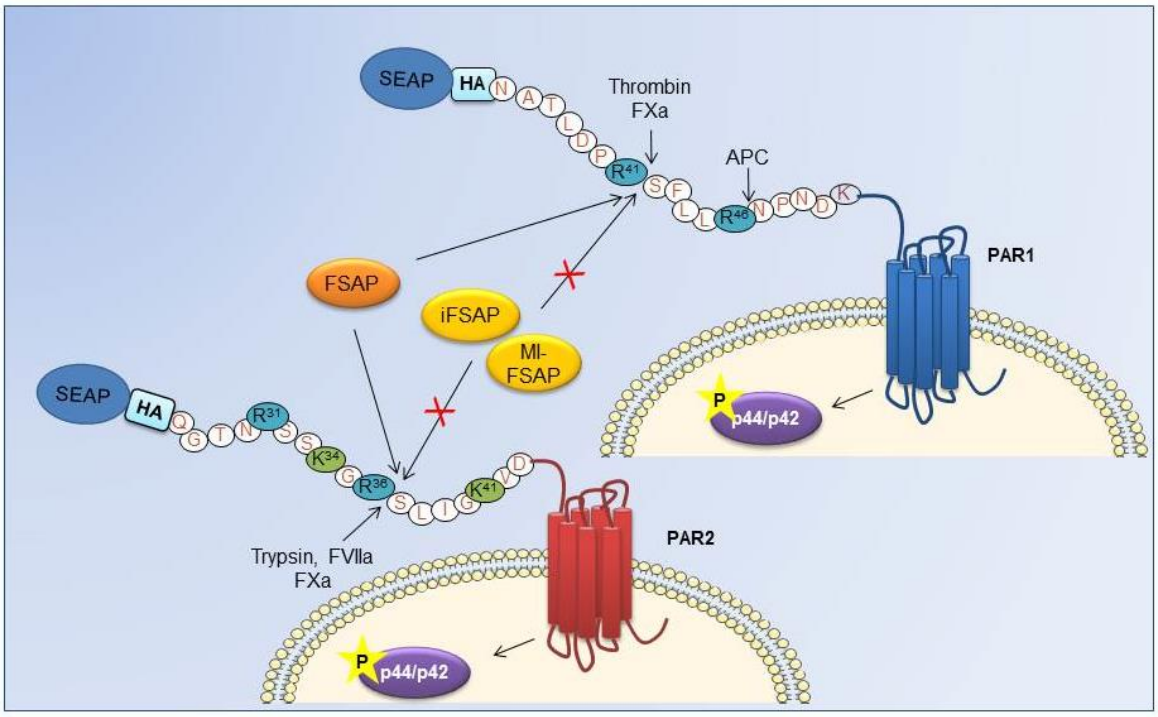

Figure 8. Graphical summary of the main finding in paper II. 1) FSAP cleaves PAR2 more than PAR1, PAR3 and PAR4 are not cleaved. 2) FSAP cleaves PAR1 and PAR2 at their canonical cleavage sites. 3) FSAP activates MAPK pathway in cells expressing PAR1 and PAR2. 4) Inactive FSAP and Marburg I FSAP does not activate PARs

4 Discussion

4.1 Methodological considerations

In the first part of the discussion I will describe some of the key methods used in the studies and briefly discuss their advantages and disadvantages.

4.1.1 Cell cultures

For studying biological processes, cell cultures have many advantages over *in vivo* animal studies in term of ethics, simplicity of experimentation and costs. Cell cultures are very well defined systems, and it is easy to control all the experimental conditions. On the other hand, cell cultures are isolated systems and do not mimic the complex interrelationship of the *in vivo* settings. In principle there are two categories of cell cultures; primary cell cultures and cell lines. Primary cells are harvested directly from an animal or humans and brought into culture. Primary cells are closer to *in vivo* settings than cell lines, but they also have limitations. Primary cells have short life span, they lose their phenotype during cultivation, and it is difficult to manipulate gene expression in primary cell cultures. Furthermore primary cell cultures are rather heterogeneous population compared to established cell lines. Established cell lines are very homogenous. They have been derived from tumors, immortalized or transformed to allow continuous proliferation in culture. Cell lines are very well defined regarding gene and protein expressions and they are normally quite easy to manipulate through gene knock-in, knock-out or gene editing. This makes cell lines a valuable tool to study mechanisms under controlled conditions although established cell lines can be phenotypically very different from the cell type they represent.

In paper I we use primary cell cultures, human coronary artery smooth muscle cells (CASMC) and human umbilical cord vein endothelial cells (HUVEC) to study FSAP mediated gene expression and secretion of proteins. In paper II we used established cell lines namely; Human embryonic kidney cells (HEK293T) and human lung adenocarcinoma epithelial cells (A549). The usage of these cell lines allowed us to over-express tagged-proteins, and thereby study gain-of-functions.

4.1.2 Detection of global gene expression and RT-qPCR

Microarray based expression profiling is a fast and fairly accurate method for determining global gene expression patterns. Currently, this is superseded by high throughput sequencing that not only gives information about expression levels and alternative splicing but also other forms of RNA e.g. miRNA and long non-coding RNA are quantified. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was used to verify these results. It is sensitive, stabile and confers high reproducibility. Two main types of RT-qPCR methods exist, probe based and SYBRGreen. The first step is conversion of RNA into cDNA through reverse transcription. Probe based systems like e.g. Taqman, contain a forward and reverse primer and a quencher which binds to the cDNA. Upon polymerization of the cDNA the quencher is released which releases a fluorescent signal. Due to the quencher, non-specific off targets amplicons will not give a signal, and the system is therefore very reliable. SYBRGreen releases a fluorescent signal when bound to double-stranded DNA. This means that even non-specific amplification will give a signal. PCR are carried out for 35-40 cycles and by the end of each polymerization, the fluorescent signal is detected, and relative expression can be quantified.

We have used the SYBRGreen method and each primer pair was carefully designed and tested. Since contaminating genomic DNA is present in almost all cDNA preparations, primers sets spanning at least one exon-exon boundary, were used to exclude genomic DNA amplification. Primers were designed to give short products in the range of 70-200bp and were verified on agarose gels. The efficiency of all primer sets was tested, making the system as reliable as possible. Results from RT-qPCR analysis are most often calculated using ΔΔCT method. This method implies a 95-100% efficiency of each primer pair which is rarely the case. The studies on gene expression presented here have been calculated taking efficiency into account as described by Hellemans *et al*.[164]. Lastly, the choice of a reference gene is important. No globally available housekeeping gene exists, and for each type of experiment the suitability of the reference gene must be evaluated. Throughout our studies we have been using GAPDH as a reference gene, however we have tested

both beta-2-microglobulin (β2M) and ribosomal protein L19 (RPL19), and both gave similar results as GAPDH in the results presented here.

4.1.3 Detection of protein expression

The three methods used are 1) Western blotting, 2) enzyme-linked immunosorbent assay (ELISA) and 3) immunofluorescence staining of cells. All three methods are antibody based, sensitive and specific.

For western blotting proteins were separated by sodium dodecyl-sulphate polyacrylamide gel electrophorese (SDS-PAGE) according to size and transferred to a high binding capacity polymer membranes. The proteins were detected using specific primary antibodies followed by washing and incubation with secondary antibodies conjugated with an horse radish peroxidase (HRP). HRP signal were detected in conjunction with enhanced chemiluminescence (ECL) detection system. Densitometry analysis of the bands of interest and comparison with loading controls, allows semi-quantitative analysis.

In ELISA primary capturing antibody is immobilized. The sample/proteins are allowed to bind to the coated antibody. After washing away unbound proteins, a detection antibody linked to biotin is bound to the proteins. The final two steps are binding of streptavidin-HRP and detection using a substrate which develops color. Absorbance is released and equated to standards.

For immunofluorescence staining, cells are seeded on a coverslip and fixed to keep architecture and proteins at a fixed position. Depending on the fixative the antigenicity of the proteins is altered in different ways. The cells can then be either permeabilized, which allows to detect intracellular proteins or non-permeabilized to detect only membrane located proteins. By using primary antibodies raised in different species, and secondary antibodies with different fluorophore linked, detection of different proteins on the same slide is allowed. In paper II cells were fixed using paraformaldehyde, and permeabilized using 0.1% Triton X-100. Transgenic protein expression was verified and slides counterstained with the DNA stain, 4',6-diamidino-2-phenylindole (DAPI). Immunofluorescence staining gives

information about the localization of the proteins in the cells and it gives us an idea of transfection efficiency.

Stained cells were analyzed by epifluorescence microscopy. This detects the entire signal given by a fluorophore at different levels within the sample. In contrast, confocal microscopy detects signals layer by layer, which allows a much more exact localization of the signal.

4.1.4 Inhibition of receptors and signaling pathways.

For studying signaling pathways a range of small chemical antagonists for various pathways were used. Dimethyl sulfoxide (DMSO) was used to dissolve the inhibitors. DMSO can both decrease or increased inflammatory responses such as ROS production and IL-6 secretion[165, 166]. Therefore DMSO control was used to exclude any artefacts.

Antagonist selectivity can also be a problem. In paper I we use two different PAR1 inhibitors, SCH79797 and vorapaxar. Both of them block the activation of PAR1 by the tethered ligand, but not the proteolytic cleavage. This means that proteolytic cleavage of PAR1 leading to trans-activation might not be antagonized. The exact binding of SCH79797 to PAR1 is not known but vorapaxar binds to PAR1 in a rather superficial pocket but unexposed to the solvent, which makes the binding irreversible[160]. Parmodulins which selectively block down-stream effects of PAR through blocking of G-protein coupling[161] are not yet commercially available.

Other possibilities to study loss-of-functions of receptors are through RNA interference using siRNA or shRNA or through usage of neutralizing antibodies. For the studies presented in paper I both approaches were tested. Neutralizing antibodies have been developed for PAR1 against different epitopes, ATAP2 recognizes the thrombin generated tethered ligand and thereby blocks thrombin mediated-activation of PAR1, WEDE12 blocks the hirudin like domain of PAR1, and thereby competes with thrombin binding to PAR1[141]. We found non-specific effects of these antibodies thus we could not use them in this study. Regarding the siRNA approach, the transfection reagents by itself greatly affected viability of the cells and

influenced the expression of all reference genes tested. Thus, this approach was not pursued further as well.

4.1.5 SEAP reporter system

In Paper II we have studied the cleavage and activation of PARs using a secreted alkaline phosphatase (SEAP) reporter system. Mammalian expression vectors containing an N-terminal SEAP-tag followed by a HA-tag and PAR1, 2, 3 and 4 coding sequences, respectively, were transfected into HEK293T or A549 cells. For our experiments we used transient transfections to avoid artefacts arising from using clonally expanded cells that might exhibit anomalous behavior. Using transient transfections, based on optimization of DNA content, transfection conditions and transfection time, optimal expression can be obtained and therefore provides an effective tool for short experiments. Over-expression of proteins can lead to artefacts in that interactions occur that may not be possible when normal levels of these proteins are expressed.

4.1.6 Detection of cleavage sites

To study FSAP cleavage sites in PAR1 and PAR2 two methods were used; introducing point mutations in the SEAP-PAR constructs and cleavage of a synthetic peptide followed by mass spectrometry. The transgenic proteins would be expected to fold as endogenous proteins and the FSAP-mediated cleavage and signaling therefore mimics a more natural setting. The mutations introduced into PAR1 and PAR2 was made based on the assumption that FSAP would cleave after one of the basic amino acids around the canonical cleavage site. The use of synthetic peptides allowed a hypothesis free approach and would therefore detect other potential cleavage sites as well. However, synthetic peptides are short and cannot be expected to fold in a natural way. The combined usage of these two methods adds more validity to our results.

4.2 General discussions.

The focus of our studies was to characterize the changes in gene expression pattern in VSMC and EC after stimulation with FSAP. Based on the changes in gene expression as well as evidence from the use of specific inhibitors we concluded that a PAR-dependent mechanism was involved. This was further consolidated through the use of PAR-activated reporter assay and experiments with synthetic peptides which confirm that PAR1 and PAR2 are bona fide receptors for FSAP. The identification of the receptors for FSAP provides a strong basis to further define the role of FSAP *in vivo* and expand its potential for therapeutic purposes.

4.2.1 Gene expression pattern in FSAP-stimulated cells and its role in inflammation.

In paper I we show a panel of genes which were up-regulated by FSAP. Pathway analysis of the up-regulated genes in EC was primarily pro-inflammatory genes (*SELE, VCAM1, CXCL1, ICAM1 and IL8*) whereas genes related to proliferation (*AREG, EREG, HBEGF*), apoptosis (*BDNF, EDNRB, DUSP1, MEF2C*) and inflammation (*IL6, CCL2, LIF* and *PTGS2*) were up-regulated in VSMC. Down-regulated genes belonged to aldo-keto reductase for VSMC and mitosis, cell division, and cytoskeleton organization for EC. These results are similar to those obtained earlier in monocytes by Parahuleva *et al.*[87], which also show a predominantly inflammation-related gene expression pattern. It was demonstrated that the lack of endogenous FSAP, in $FSAP^{-/-}$ mice, seems to promote an inflammatory state by recruiting leukocytes into areas of tissue injury and remodeling[75]. However, exogenously added FSAP induced a pro-inflammatory response in both VSMC and EC. Thus, the *in vitro* results are very surprising and counter-intuitive to what has been shown in knock-out mice. FSAP seems to promote, both, pro-inflammatory effects *in vitro* and anti-inflammatory effects *in vivo* depending on the test system. *IL-6* and *PTGS2* which is regulated by FSAP are traditionally considered pro-inflammatory[167]. However, inhibition of PTGS2 leads to more atherosclerosis[168] and blocking *IL-6* also revealed a role in inhibiting metabolic inflammation[169]. Thus, the induction of *IL-6* and *PTGS2* in VSMC *in vitro* may in fact account for anti-inflammatory effects of FSAP that are evident *in vivo*. Thus, the pro- and anti-

inflammatory effects of FSAP and the differences *in vitro* and *in vivo* need to be evaluated in a context-dependent manner.

Could FSAP have multiple effects on different cell types; some of which are pro- and some anti-inflammatory? PAR1 is known to have dual functions, it is responsible both for pro- and anti-inflammatory effects at the same time. Thrombin stimulates apoptosis in EC via PAR1, but another protease APC is a potent inhibitor of apoptosis through cleavage at a non-canonical site in PAR1[163]. Correspondingly, stimulation of PAR1 can lead to vasodilation or vasoconstriction in the vasculature depending on whether the target cells are EC or VSMC[170]. Targeting different receptors with the same protease can also cause biased signaling as has been observed in the case of FXa[95, 127]. A possibility, that FSAP mediates biased signaling via PARs in EC remains, but needs further investigation.

4.2.2 Proteolytic activity of FSAP is required to mediate its cellular effects.

Both, the alteration in gene expression and PAR cleavage and signaling were dependent on the proteolytic activity of FSAP. First, the effects of FSAP were inhibited by the serine protease inhibitor aprotinin and the pre-inhibited PPACK-FSAP was not able to stimulate cells. Second, recombinant WT-SPD of FSAP mediated a similar response as plasma purified full length FSAP, but this was not the case for the MI-SPD isoform which had a single amino acid mutation only. Some of the effects of the recombinant proteins did not show the expected pattern i.e. MI-SPD induced *VCAM1* gene expression to the same extend as WT-SPD and both WT-SPD and MI-SPD did not increase *AREG* expression as was shown with plasma purified FSAP. The reasons for this are not immediately clear but different genes might be regulated through different mechanisms. It was shown that coagulation factors can have different specificities towards different substrates. One amino acid change in FVIIa decreased proteolytic activity towards PAR2 but retained normal activity towards its other substrates FX and FIX[171] and mutating three lysine to three alanine residues in APC completely removes its anti-coagulant activity but retains cell signalling capacity[163]. It is possible that a similar mechanism is true for FSAP which would explain why MI-FSAP can increase *VCAM1* gene expression in EC but not the other

genes studied. However, this proposal requires further validation. Thus, in general a proteolysis-based cellular signalling mechanism of FSAP seems likely.

4.2.3 FSAP signaling is mediated via multiple cellular receptors?

FSAP has been shown to cleave PAR1 and PAR3 in pulmonary EC and FSAP-induced changes in endothelial permeability can be inhibited by silencing PAR1 and PAR3 by either siRNA or neutralizing antibodies[86]. Similar observations have been reported in astrocytes and neurons[74]. Using small MW inhibitors we have shown that the effects of FSAP on VSMC are mediated via PAR1 and not PAR2 and that induction of gene expression in EC by FSAP is not mediated by PAR1. In the over-expression system we found that FSAP activates predominantly PAR2, to a lesser extent PAR1, and PAR3 and PAR4 not at all. The different conclusions may be related to the use of different cells and read-outs. Earlier studies have suggested that FSAP activate bradykinin- and FGF2-dependent signalling pathways in EC[172]. It can therefore not be excluded that these pathways are responsible for the effects of FSAP on gene expression in EC.

The activation profile of PAR receptors is determined by various factors; cellular localization in lipid microdomains in the plasma membrane, proximity to co-receptors and adapter molecules, expression pattern of PARs and their ability to dimerize with each other, expression of particular G-proteins as well as β-arrestin that drives cellular signaling[163, 173-176]. FSAP can activate PAR1 on VSMC and pulmonary EC but not on HUVEC, probably, because an additional cofactor is missing on the latter cell type. In the PAR over-expression system heparin promoted the cleavage of both PAR1, 3 and 4 by FSAP although only PAR1 cleavage is statistically significant. These results strongly suggest that FSAP-mediated PAR1 cleavage is dependent on a co-receptor or the cross-linking effect of heparin. The effect observed by heparin is not due to activation of pro-FSAP, since the majority of FSAP preparations contains active FSAP, rather heparin may be bridging FSAP, PARs and some co-receptors. In VSMC or EC no effects of heparin on FSAP-mediated gene expression was observed (data not shown).

Various studies have shown that PAR1 can trans-activate PAR2[115-118, 149].We examined the possible role of receptor dimerization between PAR1 and PAR2 by

over-expressing SEAP-PAR1 and PAR2-Flag in HEK293T or A549 cells but did not find any evidence that this leads to a major change in either the activation or cleavage of these receptors (data not shown). Similarly, some Gla domain-containing proteases such as APC and FXa need the co-receptor, EPCR to facilitate PAR activation[98, 122]. No change in the activation profile of cells was observed, when EPCR and PAR1 were co-expressed in cells indicating that it is unlikely that EPCR is involved in the signaling pathways activated by FSAP.

In the inhibitor screen with various generic pathway inhibitors (LY294002, SB203580, UO-126, PD98059, RP-8-Br-cAMPS and ML-7), a strong inhibitory effect of a cAMP antagonist (RP-8-Br-cAMPS) on FSAP-mediated gene expression in VSMC was identified. This is in line with the fact that thrombin and angiotensin II, were shown to stimulate *IL-6* expression in VSMC through the CRE[147, 177]. In addition, CRE binding protein (CREB) phosphorylation in response to FSAP has been demonstrated before in EC[172]. We also investigated if the CRE binding protein (CREB) was activated by FSAP in VSMC. An increase in FSAP stimulated phosphorylation of CREB was observed although this was not statistically significant (Figure 9). The inhibitory effect of the MAPK-p44/p42 inhibitor PD98059 was in line with earlier observations that there is a weak activation of MAPK-p44/p42 by FSAP in VSMC[54]. There was also an increase in phosphorylation of MAPK-p44/p42 but again this was not statistically significant due to high variation in the levels of activation between experiments (not shown).

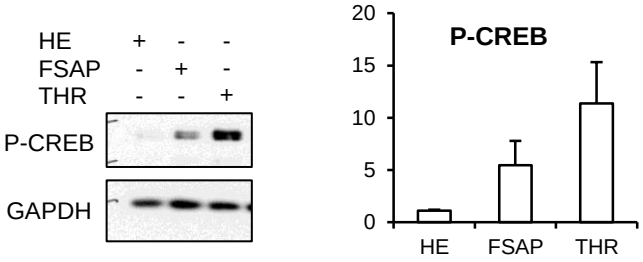

Figure 9 FSAP activates CREB signaling in CASMC. CASMC were stimulated with FSAP (10 µg/ml), thrombin (1 U/ml) or control HE buffer for 15 min. Activation of CREB were determined using western blotting. Densiometric analysis represent ratio of phosphor-CREB vs GAPDH. Data represent mean of 3 independent experiments ± SEM. These changes are not statistically significant.

Another possible mediator in the FSAP-induced pathway could be EGFR. EGFR trans-activation was described for thrombin induction of *IL-6*[147] in VSMC. In

our studies, inhibition of EGFR with the inhibitor, AG1478, led to a decrease in *IL-6* and *AREG* gene expression (not shown), but we were unable to detect phosphorylation of the EGFR by western blot. It was recently shown that 50 % of all genes, e.g., *IL-6, PTGS2* and *AREG* regulated through PAR1 activation are dependent on trans-activation through either EGFR or TGFBR1[152]. It therefore seems likely that the regulation of these genes by FSAP also involves trans-activation of EGFR.

4.2.4 The FSAP cleavage site(s) in PARs:

By introducing point mutations in the N-terminal changing the lysine and arginine residues to glycine we identified the non-canonical, K_{34} and canonical, R_{36} sites in PAR2 as being potential cleavage sites for FSAP since both mutations abolished SEAP release and signaling. Synthetic PAR2 N-terminal peptide was only cleaved at the canonical R_{36} site.

It is well established that the amino acids in the P1-P4 positions are important for protease to bind and cleave their receptor/substrate. The amino acids in these positions as well as amino acids in exo-sites confer specificity for ligand binding and cleavage. FXa cleavage knock-outs of PAR2 were engineered where a mutation at the P2 position of PAR2 abolished FXa cleavage but retained the ability to be cleaved by FVIIa and Trypsin[171] all sharing the canonical cleavage site. Others have showed that by interchanging P2 and P3 residues of PAR1 and PAR2, selectivity for thrombin and FXa cleavage and activation were interchanged as well[178]. New results from our group on substrate specificity for FSAP show that FSAP prefer a basic cluster in the non-prime side of cleavage site[58]. Therefore it is not surprising that the K_{34}G mutant completely abolished FSAP cleavage and signaling. By changing the K_{34} at position P3 we might disrupt this basic cluster and thereby possibly lose binding and cleavage of the receptor by FSAP.

We also mutated the canonical R_{41} and non-canonical R_{46} in PAR1, both resulting in an inactive receptor. FSAP cleaved the synthetic PAR1 peptide at canonical R_{41} only. We cannot immediately explain our observations on the R_{46} mutant and this warrants further investigations.

4.2.5 Translation of *in vitro* results into *in vivo* context:

FSAP protects the vascular wall against neointima formation after vascular injury[66]. Furthermore FSAP$^{-/-}$ mice showed increased neointima formation after vascular injury compared to WT animals[75]. Proteolytic inhibition of PDGF-BB derived proliferation and migration in VSMC[54] or increased MMP2 and MMP9 activity together with increased leukocyte infiltration[75] were proposed as potential causes for the FSAP mediated effect. In the studies presented in paper I the gene expression pattern identified proliferative genes to be induced by FSAP. Expression of the mitogens *AREG* or epiregulin (*EREG*) would increase proliferation by VSMC although a substantial increase in cell proliferation was not observed. It is currently not clear how to place the *in vitro* results in an *in vivo* context but the presence of different cells and factors *in vivo* might account for the differences observed.

Apoptosis is involved in vascular pathological conditions involving VSMC proliferation like atherosclerosis and restenosis. Since apoptosis-related genes were also up-regulated in VSMC we therefore also studied the effect of FSAP on apoptosis in VSMC. FSAP decreased the level of apoptosis after serum starvation in VSMC (Figure 10A) and this effect were completely reversed by pre-treatment of FSAP with aprotinin (Figure 10B). FSAP expression was increased in unstable atherosclerotic plaques[44] in humans. In relation to the role of FSAP in apoptosis, FSAP would tend to promote the retention of VSMC within plaques and increase stability of the plaque, and thereby have a beneficial role on vascular pathophysiology.

Even though the pathogenesis of neointima formation and atherosclerosis both involve VSMC proliferation, the role of FSAP in the two conditions might be different and the mechanism of action might be what determines the effect; One suggestion could be a) an anti-proliferative function driven by PDGF-BB after vascular injury, and b) a PAR1 or PAR2 mediated pro-inflammatory and anti-apoptotic effect during e.g. atherosclerosis. It is however important to remember that what we observe here is in an isolated cell system where we control all the factors added to the system and other mediators of the FSAP-mediated effects cannot be out ruled.

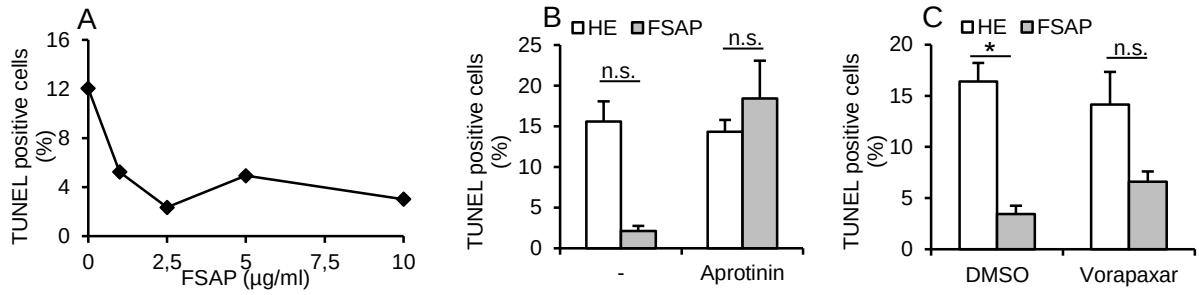

Figure 10 FSAP protects CASMC against apoptosis. CASMCs were serum starved for 16 h in the presence of FSAP (10 µg/ml) or HE buffer and apoptosis were measured using TUNEL staining. Percentage of TUNEL positive cells compared to DAPI stained cells are listed. When FSAP were pre-treated with Aprotinin (25 µg/ml), the protective effect of FSAP on apoptosis were lost. Pre-treatment of the cells with 1µM Vorapaxar partially reversed the protective effect of FSAP on apoptosis. Data represent mean of 3 independent experiments ± SEM. * indicate $p<0.05$ using one-way ANOVA followed by TUKEY's multiple comparisons test.

4.2.6 FSAP and PAR2 in lung carcinoma epithelial cells:

It has been shown that, both, PAR1 and PAR2 are expressed in various lung cancer cell lines such as A549 and PC-14 and lung cancer tissue[179]. Activation of PAR1 increased cytokine secretion[180] and activation of PAR2 increased cytokine release as well as expression of inflammatory proteins such as PTGS1 and PTGS2[180, 181]. Interestingly, a correlation between FSAP expression and lung disease was recently suggested by Mirzapoiazova[45] and Wygrecka[182] and FSAP expression was upregulated in various lung cancer tissue samples and cell lines[45, 182]. Mirzapoiazova and coworkers showed that up-regulation of FSAP expression in a lung epithelial cancer cell line increase uPA activation, cell migration and trans-endothelial extravasation[45]. Further studies are required to test whether FSAP induces pro-inflammatory gene expression or suppresses apoptosis in these cells as we have shown on VSMC. Preliminary studies suggest a suppressive effect of FSAP on apoptosis in A549 cells as well (not shown) but this need to be confirmed. In this context the findings from Gara *et al*. relating to the clonogenic proliferation of cancer cell lines after over-expression of FSAP[78] may also be important.

5 Conclusion and future perspectives

The Marburg I polymorphism of FSAP has proven to be a valuable tool to identify the possible role of FSAP in diseases. Although many conditions have been identified with a potential involvement of FSAP there is still a lack of mechanistic information. In the investigations presented in this thesis we have gained further insight into the cellular functions of FSAP. Global mRNA expression analysis revealed that FSAP mediated a pro-inflammatory response in VSMC and EC similar to thrombin. The proteolytic activity of FSAP was important for this response and PAR1 mediated the responses in VSMC but not in EC. Our preliminary findings suggest that FSAP, in addition to regulating inflammation pathways, also harbors a cytoprotective effect. In future investigations we want to study the cytoprotective effect of FSAP in more details, we want to know which receptors mediate this effect and through which mechanism. A number of down-regulated pathways were also identified in these experiments and these need to be further analyzed in order to gain a complete overview about the actions of FSAP on vascular cells. Furthermore, the inflammatory nature of the induced genes show that a similar analysis needs to be performed on monocytes.

Using a sensitive PAR cleavage reporter system, we show that FSAP directly cleaves PAR1 and PAR2 at the canonical trypsin cleavage site and that this cleavage mediates activation of MAPK-p44/p42 signaling pathway. The effects of PAR activation are very much dependent on the cellular context, expression of inflammatory genes and cytoprotection in the epithelial cells lines we studied, needs further detailed investigations. The experimental system used here is amenable to further manipulation in that co-receptors or PAR-PAR interactions, PAR-internalization and coupling to intracellular signaling pathways can be investigated.

The interaction between FSAP and PAR signaling in lung cancer would be interesting to explore because these cells also express FSAP endogenously indicating an autocrine loop in these cells. In all our studies MI-FSAP did not have any influence on gene expression nor did it activate any of the PARs. This fits into the general pattern that this mutant is enzymatically inactive. This alludes to the fact that FSAP normally has a protective function in the body which is absent in MI-FSAP. This could be related to the cellular effects of FSAP described here.

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