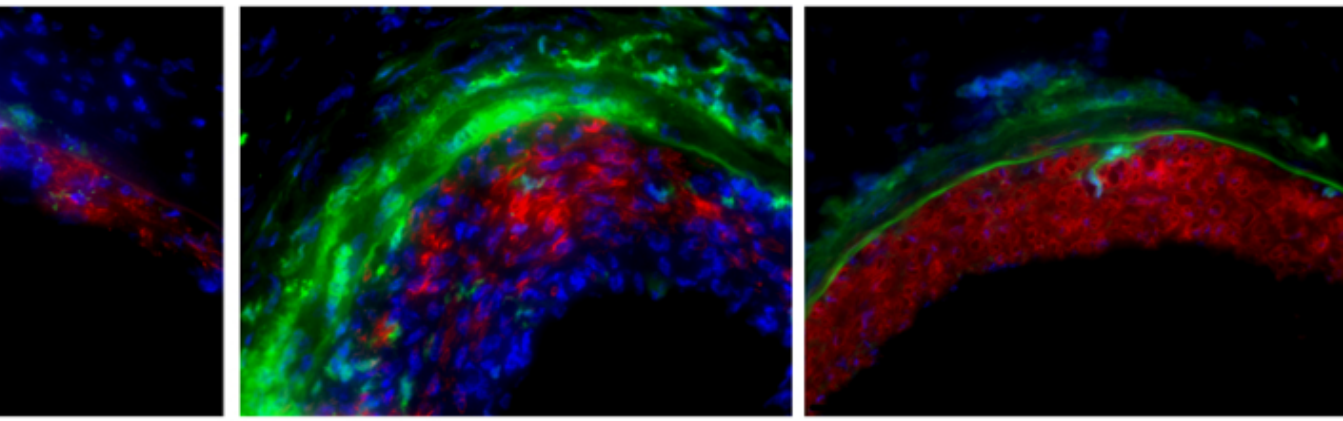
The *in vivo* effects of the factor VII–activating protease (FSAP) on neointima formation



Inauguraldissertation
zur Erlangung des Grades eines
Doktors der Medizin
des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

vorgelegt von
Jan-Marcus Daniel
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Aus dem Institut für Biochemie der
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PD Dr. Sawa Kostin

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09.06.2010

Für meine Eltern

Ute und Hans-Werner Daniel

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1. Introduction

Cardiovascular diseases due to atherosclerosis are the leading causes of death in the western world[1, 2]. The current strategies to prevent atherosclerotic lesions aim to reduce the major risk factors, e.g. hypertension or diabetes. Genetic risk factors also play a major role in the development of vascular proliferative diseases, but the pathophysiology of most of these genetic aberrations is poorly understood[3]. Factor VII activating protease (FSAP), a novel plasma protease, has been shown to be linked to vascular diseases in humans, since the Marburg I (MI, G534E) polymorphism of FSAP is a prominent risk factor for atherosclerosis and stroke[4, 5]. In the Bruneck study, a prospective population based clinical survey, the odds ratio of advanced atherosclerosis in homozygous MI-patients was 6.63 (1.58-27.72) and exceeded even the risk profile for diabetes mellitus[4]. In the European population, there are ~5% of heterozygous carriers of the MI-polymorphism, and further clinical studies are on the way to investigate the risk profile of the affected patients. However, it is not known how the gene is involved in the disease process. On the basis of our *in vitro* studies, we therefore aimed to elucidate the complex role of FSAP in the pathogenesis of vascular diseases *in vivo*.

1.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arterial wall and can result in coronary heart disease, stroke, or aneurysm formation[6]. The major risk factors are hypertension, diabetes mellitus, hypercholesteremia, and smoking[7]. These factors cause an endothelial dysfunction, which is characterized by a shift toward reduced vasodilatation, prothrombotic properties, and a proinflammatory state in general[8]. Low-density lipoproteins (LDL) infiltrate the arterial intima and are retained in the endothelial layer. Oxidation or enzymatic modification of LDL leads to an activation of the endothelium by bioactive lipids[9]. Particularly at sites of hemodynamic shear stress, the endothelial cells (EC) express adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1 or intercellular adhesion molecule (ICAM)-1, and secrete pro-inflammatory mediators[10]. Thrombocytes are the first cells adhering to the activated endothelium, followed by a rolling of leucocytes along the vascular surface[11]. Predominantly monocytes and T-cells adhere to these sites and start to infiltrate the subendothelial space[12, 13]. This process of chemotactic attraction is

regulated by various chemokines produced in the inflamed intima, e.g. the monocyte chemoattractant protein (MCP)-1 /CC motif receptor 2 (CCR2) axis[14, 15] (fig.1).

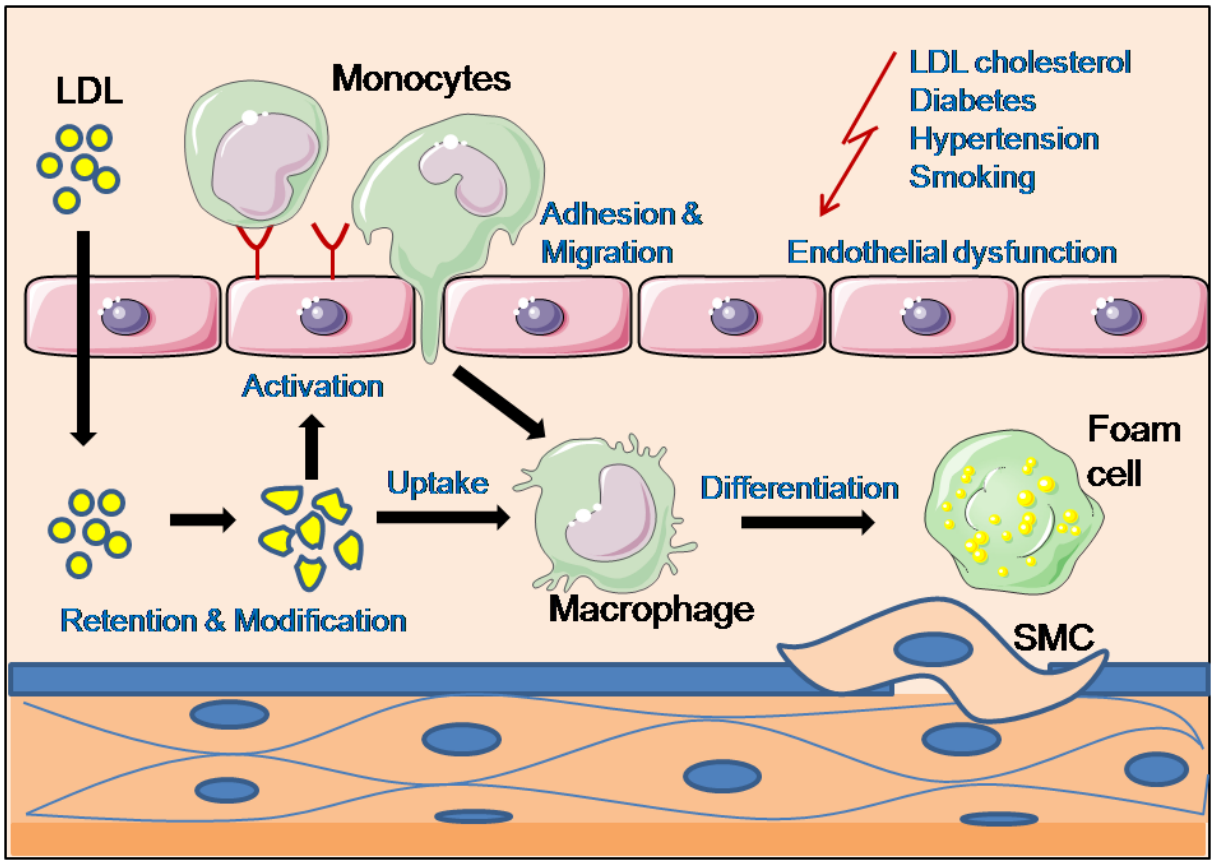

Figure 1. Infiltration of LDL and recruitment of leucocytes

Oxidative and enzymatic modifications of the infiltrating LDL are followed by an up-regulation of adhesion molecules on the endothelial layer, and thus a recruitment and transmigration of leukocytes. The modified LDL particles are taken up by macrophages, which evolve into foam cells.

The next important step for the development of atherosclerosis is the differentiation of the infiltrated monocytes into macrophages by the macrophage-colony stimulation factor (M-CSF)[16]. This step is associated with the expression of a special pattern of cell-surface receptors on the macrophages, including scavenger receptors[17]. Directed by these receptors, the activated macrophages can internalize oxidated LDL particles resulting in an accumulation of cholesterol in numerous cytosolic vesicles[18]. In the course of time, these macrophages slowly turn into large foam cells (fig.1). By ingesting more and more oxidated LDL particles, the foam cells can possibly burst and in turn accelerate the progression of the lesion[19]. This early state of atherosclerosis appears as “fatty streaks”, which are even prevalent in young people and can eventually disappear again[20] (fig. 2).

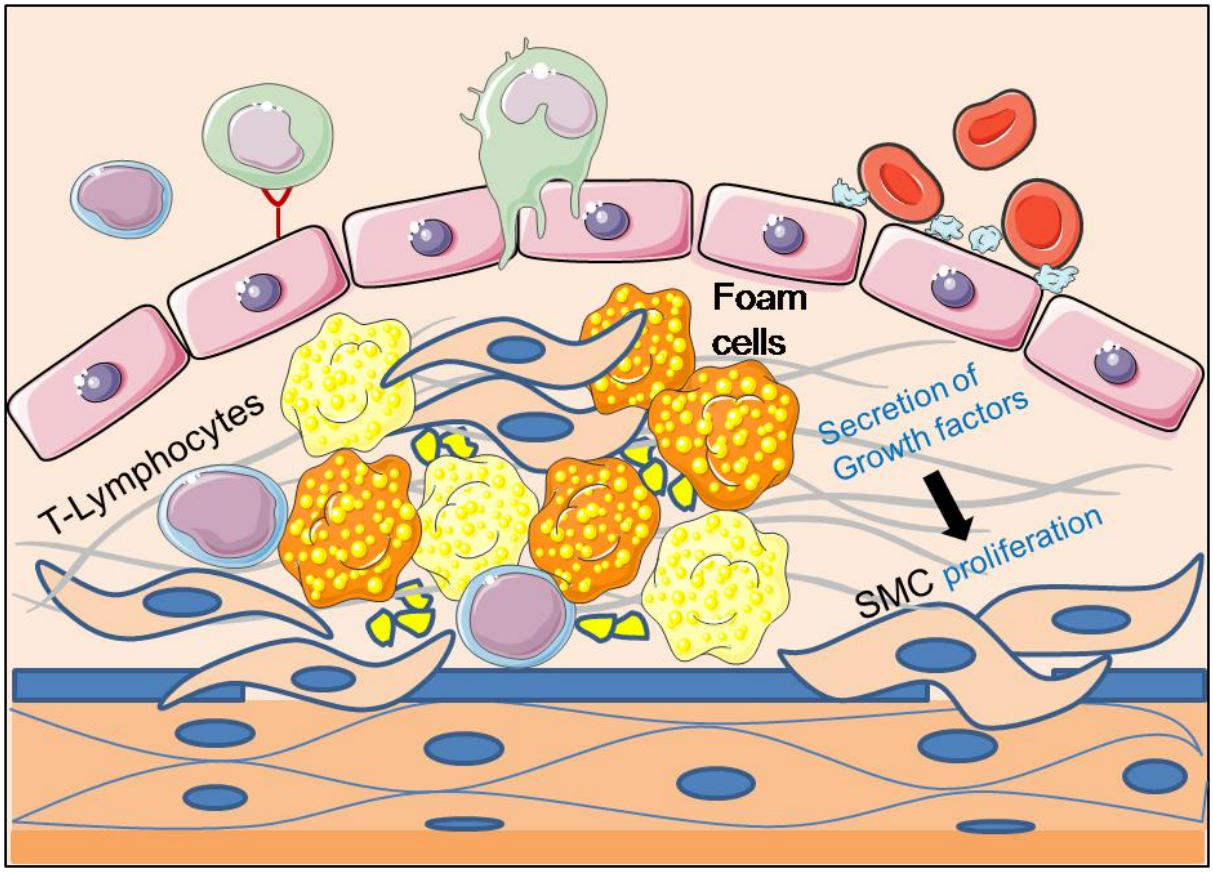

Figure 2. Fatty-streak formation during atherosclerosis

Fatty streaks mainly consist of foam cells interspersed with T-lymphocytes. These cells secrete various growth factors, including platelet-derived growth factor (PDGF)-BB and transforming growth factor (TGF)-β, to stimulate proliferation and migration of local vascular smooth muscle cells (VSMC) toward the intima, thus forming a fatty streak.

If the offending risk factors continue to be present, the fatty streaks progress to atheromata[6]. Activated macrophages or dendritic cells in the lesion fuel the inflammatory process by secreting cytokines, proteases, or by promoting oxidation[19]. Additionally, they act as antigen-presenting cells and bind via toll-like receptors (TLRs) to pathogen-like molecular patterns in the plaques, e.g. to stress proteins or DNA motifs[21]. The antigen fragments are presented by major-histocompatibility complex (MHC) class II molecules to surrounding CD4+ T cells[22]. The type 1 helper T (Th1) effector cells start to produce interferon (IFN)-γ, which improves the efficiency of antigen presentation and augments synthesis of inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1[23]. These cytokines initiate the production of large amounts of other pro-inflammatory cytokines, which in turn further stimulate the process of atherosclerosis[3]. As a result of the downstream targets in this cytokine cascade, elevated levels of interleukin (IL)-6 and C-reactive protein (CRP) may be

detected in the peripheral circulation[24]. High-sensitivity CRP, a nonspecific marker of inflammation, is even a potential biomarker for predicting the risk of cardiovascular diseases independent of other risk factors[25].

Another key process of atherosclerosis involves the proliferation of vascular smooth muscle cells (VSMC)[26]. The cytokines and growth factors, released in the intima by thrombocytes, inflammatory cells, and EC, stimulate the proliferation and migration of VSMC from the medial layer and even from the adventitia[27]. Important growth factors are amongst others platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), thrombin, and angiotensin II[26]. The quiescent "contractile" phenotype of VSMC changes to the active "synthetic" state, and the VSMC synthesize extracellular matrix (ECM) components such as collagen, elastic fibers, and proteoglycans on the luminal side of the vessel wall[28]. At the same time, the VSMC start to proliferate and migrate toward the intimal layer secreting further growth factors as well as inflammatory mediators and vasoactive substances[26]. In this fibro-proliferative state, VSMC, lipid laden macrophages, T-lymphocytes, connective tissue as well as debris from both apoptotic and necrotic cells form the lipid core of the lesion[6]. A fibrous cap is composed of VSMC and covers this core, in order to prevent contact of the pro-thrombotic material with the blood. The cap has a protective function and endows stability to the plaque[29] (fig. 3).

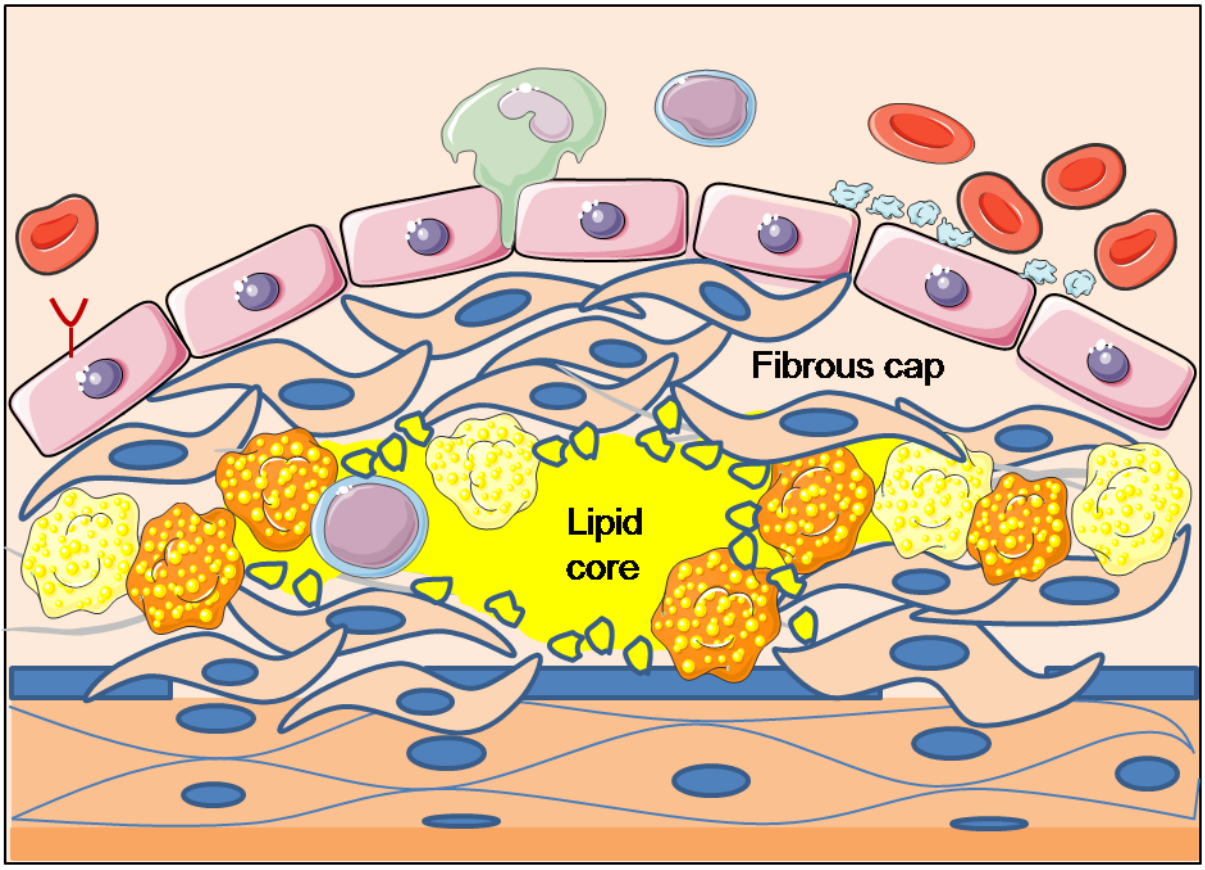

Figure 3. Formation of an advanced, complicated atherosclerotic lesion

VSMC and ECM components form a fibrous cap that covers the lipid core and protects the plaque from rupture. The lipid (or necrotic) core is highly thrombogenic and consists of a mixture of leukocytes, foam cells, modified LDL particles, and debris.

As a regulatory process, inflammatory mediators induce the production and secretion of a broad spectrum of extracellular proteases by macrophages, but also by EC and VSMC. These contain in particular cathepsin S and K, urokinase type plasminogen activator (uPA), and members of the matrix metalloproteinases (MMP) family with their tissue inhibitors of metalloproteinases (TIMP) [30, 31]. These extracellular proteinases can degrade the ECM and therefore induce thinning of the fibrous cap, thus rendering the plaque susceptible to rupture[32]. Once the fibrous cap ruptures, exposure of the pro-thrombotic content of the lipid core to the blood leads to an accumulation of thrombocytes, a thrombus formation, and finally a myocardial infarction[33]. A rupture usually occurs in the shoulder regions of a plaque, the weakest portion, where the stress is highest and the MMP-activity increased[34]. (fig. 4).
Hence, the transformation of a stable plaque into a vulnerable plaque is of particular interest in the pathogenesis of an acute coronary syndrome, and FSAP possibly influences this turnover in an essential way.

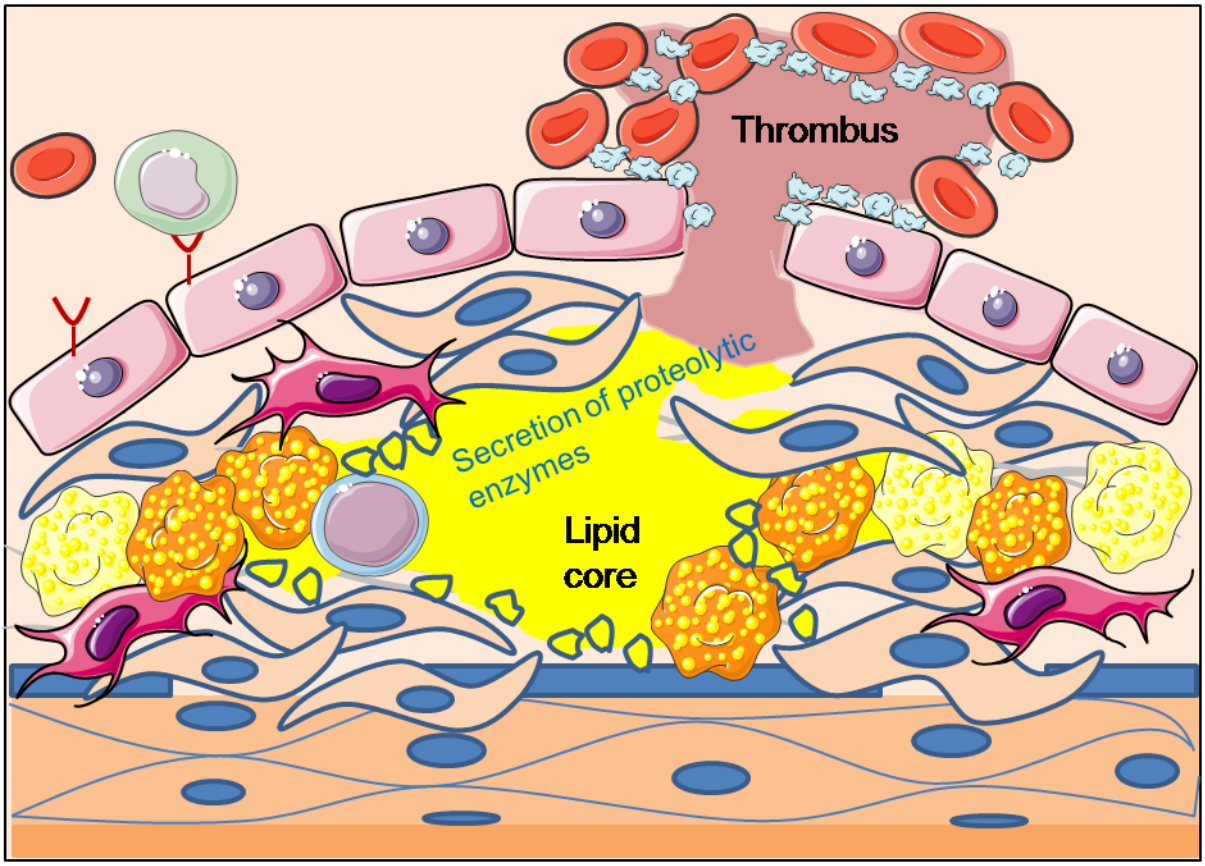

Figure 4. Thrombus formation due to plaque rupture

A ruptured fibrous cap can cause a fatal myocardial infarction. Proteolytic enzymes as well as apoptosis of VSMC are responsible for the thinning of the fibrous cap covering the pro-thrombotic material of the lipid core.

1.2 Neointima formation and restenosis

The neointima formation concerns patients after percutaneous transluminal angioplasty (PTA), bypass operation, or graft vasculopathy[35]. In cardiology, it mainly occurs after percutaneous transluminal coronary angioplasty (PTCA), e.g. for chest pain or after a myocardial infarction[36]. A catheter is inserted via the femoral artery to inflate a balloon in the affected coronary artery, compressing the plaque and dilatating the narrowed coronary artery to restore a sufficient blood flow. This procedure is typically accompanied by inserting an expandable metal stent to impede the subsequent collapse of the artery and to prop it open. Because of the unavoidable injury of the endothelium, the pro-thrombotic subendothelial components of the artery are exposed to the blood. Activated thrombocytes adhere to the vessel wall, and an inflammatory process starts by means of chemokines and growth factors, thus leading to an accumulation of leucocytes. This cascade culminates in the proliferation

and migration of resident VSMC, which secrete ECM proteins and build up a neointimal tissue[37] (fig. 5).

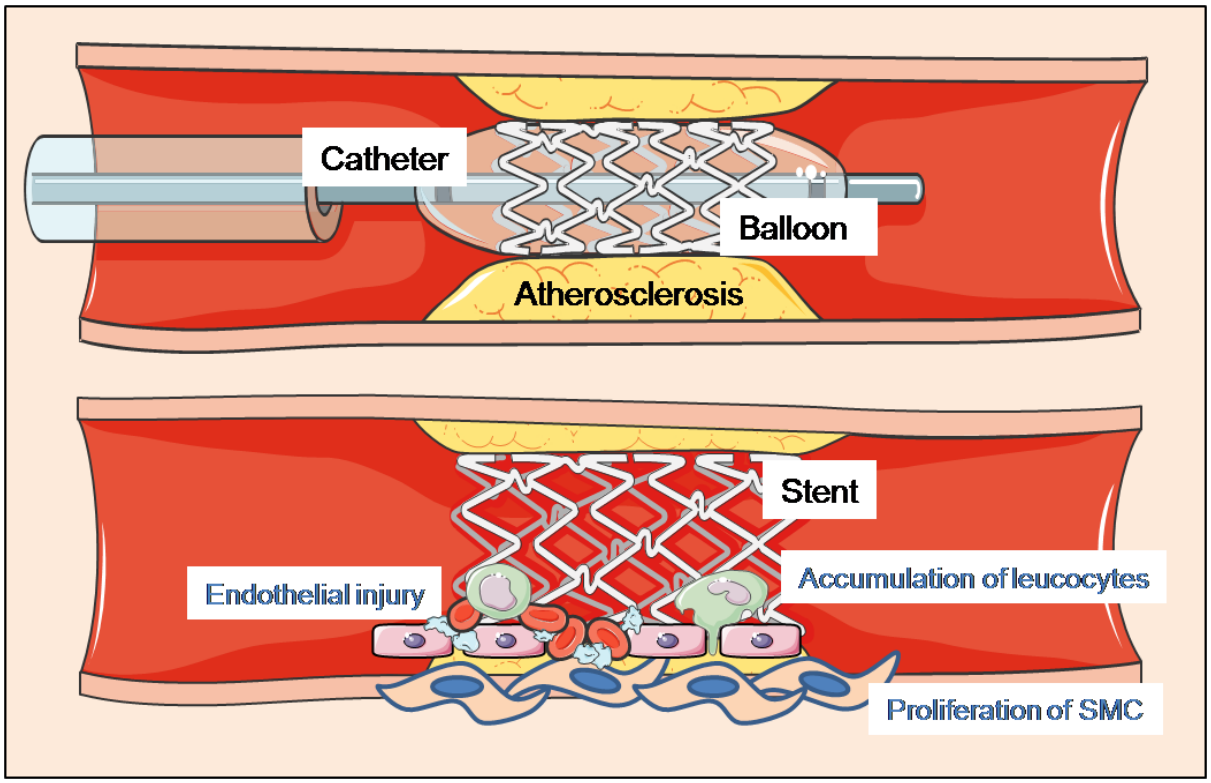

Figure 5. Balloon angioplasty and stent insertion

Dilatation of an atherosclerotic plaque is usually followed by the insertion of a stent to impede the collapse of the artery. Since the endothelium is damaged by the angioplasty, an inflammatory process is initiated. The growth factors secreted in the process initiate the proliferation and migration of medial VSMC, which possibly re-narrow the lumen of the artery.

The spreading tissue can in turn re-narrow the lumen and restrain the blood flow of the artery, so that a following PTCA is often unavoidable. Therefore, drug-eluting stents (DES) have been introduced into clinical practise to anticipate this proliferative process, and indeed the rates of restenosis could be reduced in major clinical trials from 14.3 % to 7.7 %, respectively[38]. Nevertheless, the permanent use of the more expensive drug-eluting stents is currently reassessed because of a higher rate of thromboembolic events caused by a delayed re-endothelialization[39].

A murine model of wire induced neointima formation of the femoral artery has been established by Sata *et al.* [40] (fig. 6). Until recently, it was widely accepted that intimal VSMC in vascular remodeling processes arise exclusively from resident medial cells[6]. Experiments on bone marrow (BM) transplanted mice, however, suspected circulating bone-marrow

derived progenitor cells (BMPC) of being the source of a substantial fraction of VSMC in the neointima and in atherosclerotic plaques[41, 42]. Following wire-induced injury, a significant amount of neointimal (63.0 ± 9.3%) and medial (45.9 ± 6.9%) cells were found to be of BM origin[42]. In the same publication, an analysis of atherosclerotic plaques in $ApoE^{-/-}$-mice concluded that a significant amount (42.5 ± 8.3%) of the α-smooth muscle actin positive ($\alpha\text{-sma}^{+}$) cells in the lesions were BM derived, as well. Because of a lack of high-resolution confocal microscopy analyses, this pathway remains controversial and has been challenged in mouse models of transplanted allografts and atherosclerosis induced with a collar technique[43, 44]. Furthermore, the BMPC were only shown to express α-sma, but not more specific markers for VSMC like calponin or vimentine. Hence, a transdifferentiation of BMPC into highly differentiated VSMC remains speculative.

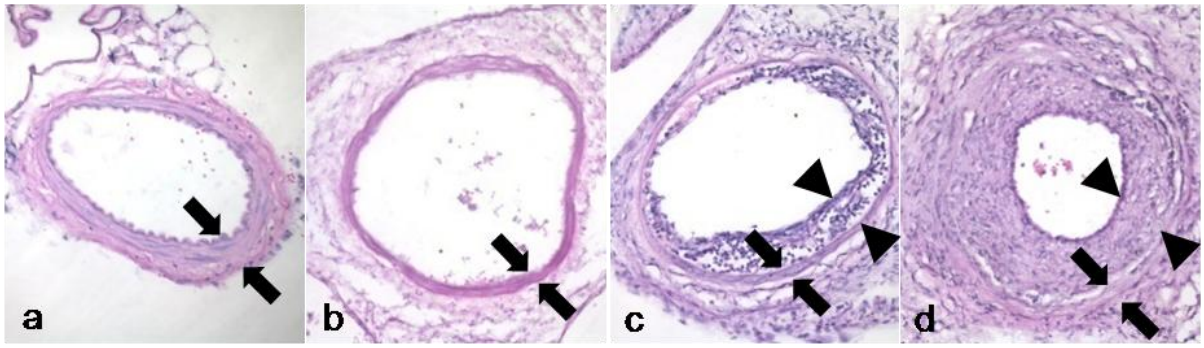

Figure 6. Neointima formation in a murine wire induced injury model

Hematoxylin & eosin (H & E) staining of a developing neointima: Uninjured artery (a), dilatated artery at 3 days with hardly any remaining medial VSMC (b), dilatated artery at 14 days with adhering leucocytes forming the neointima (c), and at 28 days after dilatation with a neointima consisting of VSMC (d). Arrows mark medial layer and arrowheads neointima formation.

1.3 Thrombus formation and hemostasis

Upon vascular injury, the hemostatic system prevents life-threatening blood loss by initiating the wound-healing process. The primary hemostasis describes the adhesion and aggregation of thrombocytes to the pro-thrombotic material of the subendothelium[45]. The glycoprotein VI and the glycoprotein Ib/V/IX complex on the thrombocytes bind to[45] von-Willebrand factor (vWF) on the ECM of the disrupted vessel and mediate the initial adhesion at arterial shear rates[46, 47]. The platelets get activated and secrete mediators such as adenosine diphosphate (ADP), thrombin, and thromboxane A_2, in order to amplify and sustain the recruitment of

circulating thrombocytes[48]. Platelet activation causes changes in the glycoprotein IIb/IIIa receptor, which binds fibrinogen molecules in order to form bridges among the platelets and facilitate thrombus formation. Since the binding of fibrinogen to glycoprotein IIb/IIIa receptor is the principle mechanism of platelet aggregation, inhibitors of the glycoprotein IIb/IIIa receptor have have been introduced into clinical practise in treating pro-thrombotic disorders[45, 49].

The secondary hemostasis is referred to as the stabilization of the platelet aggregation by the formation of fibrin. The vascular wall components (extrinsic) as well as blood-borne components (intrinsic) can initiate the coagulation cascade, leading to the production of thrombin[50]. The extrinsic pathway is initiated by tissue factor (TF, factor III), which is predominantly expressed on fibroblasts, pericytes, and VSMC in the vascular wall[51]. Factor VII from the circulation gets activated to factor VIIa and forms a complex with TF[52]. This complex in turn activates factor X to factor Xa, which forms the prothrombinase complex on membrane surfaces with its cofactor Va and activates prothrombin to thrombin. Additionally, factor VII can also be activated by thrombin, plasmin, FXIa, FXII, and FXa. The prothrombinase complex can also be initiated by the intrinsic pathway, namely factor VIIIa and its cofactor IXa, referred to as the tenase complex[50]. Hence, both pathways lead to the formation of thrombin[53] (fig. 7). The primary role of thrombin is the conversion of fibrinogen to fibrin, but it also activates other coagulation factors, the physiological anticoagulant protein C (PC), and thrombocytes[54].

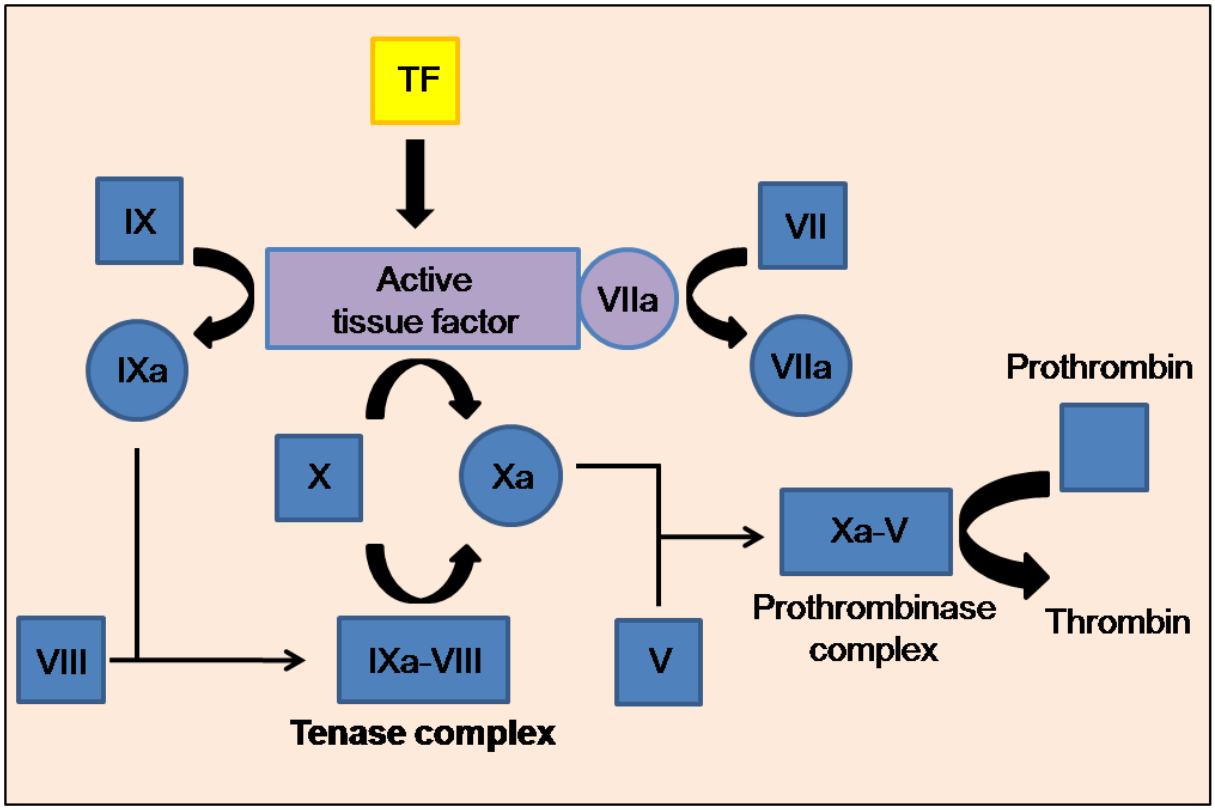

Figure 7. Pathways of thrombin activation in secondary hemostasis

The complex of tissue factor (TF) with circulating factor VIIa represents the extrinsic initiation of the coagulation system and has three substrates: factor VII, factor IX, and most importantly factor X. Factor IXa binds to factor VIIIa and forms the tenase complex, which can also activate factor X to factor Xa. Factor Xa with its cofactor Va form the prothrombinase complex and activate prothrombin to thrombin. In turn, the thrombin generated in this cascade activates the factors VIII and V, leading to a further boost in thrombin production. Picture modified after[45]

Several antagonistic mechanisms have been developed to prevent uncontrolled hemostasis. On intact EC, the important TF-factor VIIa complex gets inhibited by tissue factor pathway inhibitor (TFPI) [55]. Protein C (PC) and its cofactor protein S (PS) proteolytically inactivate factor Va as well as factor VIIIa. PC gets activated by thrombin, and this activation is further promoted by thrombomodulin, which is present on EC[56]. Furthermore, antithrombin (AT) is an important inhibitor of thrombin and other coagulation factors[57].

Taken together, the primary and secondary hemostatic systems get parallely activated by prothrombotic subendothelial material, so that the rupture of an atherosclerotic plaque leads to a thrombus formation, which in turn might result in an occlusive vessel disease, e.g. a myocardial infarction.

1.4 The plasminogen activation system and fibrinolysis

The central conversion of the plasminogen activator (PA) system is the formation of plasmin, a serine protease, which degrades fibrin to its degradation products. There are two different PA, the tissue type (t-PA) and the urinary type (u-PA) plasminogen activator. The first is synthesized by EC and then secreted to the circulation. In order to activate plasminogen, t-PA is highly dependent on the presence of cofactors, such as fibrin[58]. The concentration of uPA in plasma is lower, and it is predominantly expressed in tissues[59]. Both PA are regulated by inhibitors, most notably PA inhibitor (PAI)-1 and -2, which are present at high concentrations in plasma[60]. Additionally, uPA plays an important role in vascular remodelling, partly by activating plasminogen but also independent of this conversion[61]. The murine neointima formation in u-PA$^{-/-}$ mice is significantly impaired, and the vascular lesion induced by ferric chloride did not contain many VSMC but acellular thrombotic material[62]. In contrast, a defect in t-PA does not affect cell proliferation or migration, and the neointima of t-PA$^{-/-}$ mice does virtually not differ from that in wild-type (WT)-mice[63]. Furthermore, uPA is described to accumulate in atherosclerotic plaques, and overexpression of uPA in macrophages accelerated atherosclerosis in ApoE$^{-/-}$-mice[64]. Both functional and total uPA were increased several-fold in extracts of advanced lesions, but interestingly the uPA activity showed a high turnover in unstable plaques[65]. Since proliferation and migration of VSMC are dependent on uPA activity, a down-regulation of uPA could possibly destabilize the plaque by inhibiting the protective function of the VSMC in the fibrous cap.

1.5 Factor VII activating protease (FSAP)

1.5.1 Structure and physiology

The serine protease FSAP was originally described as plasma hyaluronan-binding protein (PHBP), when it was first purified from human plasma. FSAP is predominantly produced in the liver and circulates in plasma at concentrations of ~12 µg/ml. The structure of FSAP is composed by various structural modules, including three epidermal-growth-factor (EGF)-like domains, a kringle domain and a serine protease domain[66]. There is a high structural homology to uPA, plasminogen, or hepatocyte growth factor[67]. FSAP is produced as a single chain inactive zymogen in the liver and gets activated by cleavage of the serine protease domain from the other domains[68]. The two-chain active protease is then linked by a disulfide bond and can activate further FSAP molecules in an autocatalytic fashion[69]. In addition to its

ability to bind hyaluronic acid, FSAP was also found to have a strong affinity to other negatively charged polyanions, including mast cell derived heparin, platelet derived polyphosphates, and extracellular RNA[70, 71]. The autocatalytic activation of FSAP is particularly promoted by the interaction with these negatively charged substances. FSAP may be activated to a certain extend by uPA, but no other physiologically relevant activators have been described[68]. The activated two-chain FSAP is rapidly inhibited by various serine protease inhibitors (Serpins), including α1-proteinase inhibitor, α2-plasmin inhibitor, C1-inhibitor as well as plasminogen activator inhibitor (PAI)-1 and protease nexin (PN)-1[72]. Binding of FSAP to PN-1 leads to an inhibition of the protease, and the complex in turn binds to the low-density lipoprotein receptor-related protein (LRP) on the cell surface and gets internalized[73].

This neutralization of FSAP by various serine protease inhibitors is also promoted by contact with negatively charged polyanions[74]. Since there is a release of negatively charged anions after tissue damage, the activation and the subsequent inactivation of FSAP from the circulation is very likely to be involved in pathological processes such as atherosclerosis or neointima formation. In addition to the circulating protein, FSAP expression and FSAP mRNA were also found to be present in monocyte derived macrophages, but it is not produced by other cells in the vasculature[75].

1.5.2 FSAP in hemostasis and fibrinolysis

FSAP was named after its ability to activate factor VII independently of TF. Since exogenously added FSAP did shorten the pro-thrombin time (PT, extrinsic pathway) but not the activated partial thromboplastin time (aPPT, intrinsic pathway), the extrinsic pathway seems to be influenced by an action of FSAP[76]. Simultaneously, pro-uPA is activated by exogenous FSAP and a fibrinolytic effect is observed in whole blood[77]. The current hypothesis is that the fibrinolytic effect of FSAP is much more prominent than the activation of factor VII (our unpublished observations, fig. 8). This activation of pro-uPA could also possibly influence the uPA-mediated effects on proliferation and migration of VSMC in atherosclerosis or neointima formation.

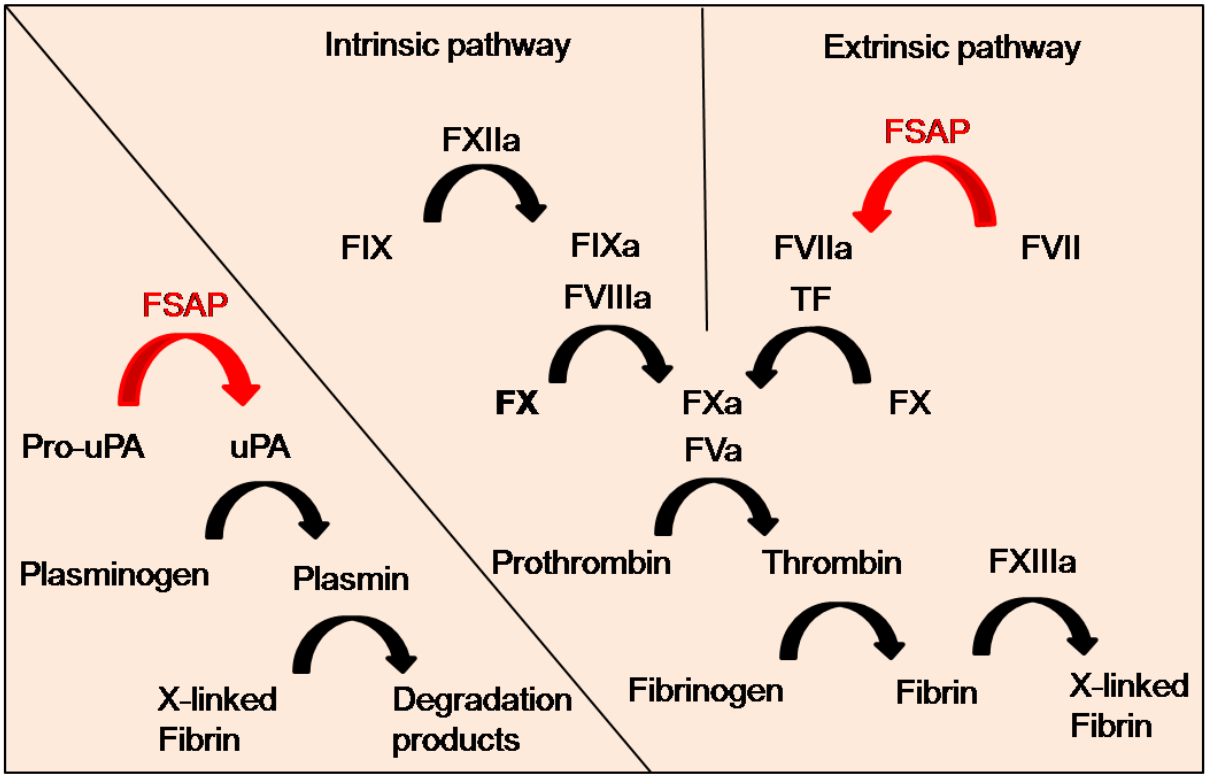

Figure 8. FSAP in hemostasis and fibrinolysis

FSAP can activate F VII independently of TF. However, our unpublished data suggest a more prominent effect on the fibrinolytic system by activating pro-uPA.

1.5.3 FSAP interaction with growth factors

FSAP has been identified as a potent inhibitor of PDGF-BB dependent VSMC proliferation and migration *in vitro*[78, 79]. This growth factor and its receptor PDGFRβ have been described in various studies of atherosclerosis and neointima formation as key players of VSMC proliferation and migration[80, 81]. FSAP binds to PDGF-BB with high affinity and can specifically cleave the growth factor in a region that is crucial for receptor binding and activation. The inhibitory effect of FSAP on PDGF-BB is significantly enhanced by heparin or extracellular RNA, whereas an inactivation of the enzymatic activity of FSAP neutralizes the effect[82]. Accordingly, the enzymatically less active MI-FSAP failed to degrade PDGF-BB, although the binding characteristics remained the same[79]. WT-FSAP also strongly inhibited PDGF-stimulated DNA synthesis, whereas the inhibition with MI-FSAP was much weaker. Similarly, PDGF-BB-stimulated phosphorylation of p42/p44 mitogen-activated protein kinases (MAPK)/ extracellular signal-regulated kinases (ERK) was inhibited by WT-FSAP but not by MI-FSAP. Apart from PDGF-BB, FSAP did not inhibit DNA synthesis or

phosphorylation of p42/p44 MAPK induced by insulin-like growth factor (IGF)-1, thrombin, sphingosine-1-phosphate (S1P), transforming growth factor (TGF)-β, basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF)[79]. In contrast to VSMC, the activation of fibroblasts by bFGF was enhanced in the presence of FSAP[83].

1.5.4 FSAP and uPA in atherosclerosis

FSAP is not present in normal vessels, but immuno-localization studies showed substantial immunostaining particularly in unstable atherosclerotic plaques in humans[78]. In a clinical study with 40 patients, FSAP immunostaining was significantly higher in plaques from patients with unstable angina pectoris and myocardial infarction compared to those with stable angina. Interestingly, the distribution of FSAP throughout the plaque was similar to that of uPA, which was predominantly associated with areas of high macrophage density. Additionally, macrophages have been shown to express FSAP protein and FSAP mRNA, and the expression was up-regulated by inflammatory mediators[75]. Since proliferation and migration of VSMC are dependent on uPA, the ability of FSAP to activate pro-uPA might regulate the activity or expression of uPA[62, 63]. The urokinase-system can activate plasminogen, and plasmin in turn activates a broad spectrum of MMP[32, 84]. These proteases generally promote migration of VSMC, and MMP-2 and MMP-9 have been constitutively up-regulated in the vessel wall after vascular injury[85].

1.5.5 The Marburg I (MI, G534E) polymorphism of FSAP

The MI single nucleotide polymorphism (SNP) of FSAP is characterized by an amino acid exchange from Gly to Glu in the serine protease domain of FSAP[86]. Approximately 5 % of the European population are carriers of the MI-polymorphism, which is a prominent risk factor for carotid stenosis as well as cardiovascular complications. In the analysis of the Bruneck Study, which involved 810 men and women aged 40 to 79 years, the homozygous allele of the MI-polymorphism was a strong and independent risk predictor of incident/progressive carotid stenosis (multivariate odds ratio [95%CI], 6.63 [1.58 to 27.72]) and exceeded even the risk profile for diabetes mellitus[4] (fig. 9).

Variable	Advanced Atherogenesis: No (n=234)	Advanced Atherogenesis: Yes (n=92)	Odds ratio (95% CI)	P
Glucose tolerance				<0.0001
IGT	20 (8.5)	16 (17.4)	3.31 (1.37–7.99)	0.0081
DM	10 (8.1)	21 (22.8)	6.38 (2.71–14.99)	<0.0001
Cigarettes per day	3.2±7.2	6.6±9.6	1.77 (1.30–2.40)	0.0003
Lipoprotein(a) >0.32 g/L	36 (15.4)	25 (27.2)	4.06 (1.83–8.96)	0.0005
Fibrinogen, g/L	2.7±0.6	2.9±0.6	1.53 (1.12–2.09)	0.0083
Marburg I FSAP polymorphism	5 (2.1)	8 (8.7)	6.63 (1.58–27.72)	0.0099
Factor V mutation	5 (2.1)	7 (7.6)	4.70 (1.19–18.55)	0.0291
Antithrombin III, %	96.3±13.0	92.8±16.4	0.74 (0.55–1.00)	0.0500
Platelet count, $\times 10^9$/L	217.4±56.5	230.3±56.6	1.32 (0.98–1.77)	0.0769

Figure 9. Risk profile of atherogenic factors in the Bruneck study

The odds ratio of the MI-SNP for advanced atherogenesis exceeded all other risk factors including elevated lipoproteins and diabetes mellitus. Values presented are mean ± SD or number (%). (Picture adapted from[4])

In another clinical trial, the MI-SNP was also determined as a risk factor for cardiovascular disease in general[5]. Concerning the relation of MI-FSAP to venous thromboembolism, the former observations of an elevated risk in MI-FSAP carriers has been challenged recently[87, 88]. *In vitro*, MI-FSAP had a lower activating capacity towards pro-uPA but an unchanged ability to activate factor VII compared to wild type (WT)-FSAP[86]. This modulation of the proteolytic activity of FSAP due to the amino-acid exchange in the active center is most likely the key to the reduction of pro-uPA activation and PDGF-BB-cleavage, but until now the *in vivo* effects of FSAP have not been explored at all.

2. Aims

The clinical findings of accelerated atherosclerosis in MI-carriers as well as the FSAP staining predominantly in unstable plaques emphasize the importance of the protease in vascular remodeling[4, 75]. However, the exact mechanisms of action of FSAP remain uncertain. On the basis of the molecular effects of FSAP *in vitro*, a mouse model of injury induced neointima formation was used to elucidate the effects of FSAP on VSMC proliferation and migration *in vivo*. The concrete aims of this project were to answer the the following questions:

1. What is the expression of FSAP in different murine tissue extracts?

2. Do WT-FSAP and MI-FSAP influence neointima formation in a wire induced injury model? Does this exogenously administered FSAP diffuse into the denuded artery?

3. How do WT-FSAP and MI-FSAP influence the pericellular proteolysis system in the vasculature?

4. What effects does FSAP have on neointima formation in $uPA^{-/-}$- and $uPAR^{-/-}$- mice?

5. What is the effect of FSAP on transdifferentiation of BM derived progenitor cells in the neointimal lesions?

3. Material and Methods

3.1 Material

3.1.1 Devices

Product:	**Company:**
ANOVA statistical package	Manugistics, Rockville, MD, USA
Autoquant Deblur 9.3	Media Cybernetics, Bethesda, MD, USA
Dissecting microscope: Leica S4E	Leica Microsystems, Wetzlar, Germany
FACS cell sorter	BD Pharmingen, Franklin Lakes, NJ, USA
Fluorescence microscope: Leica DMRB	Leica Microsystems, Wetzlar, Germany
IKA® Vibrax VXR	IKA® Works Inc., NC, USA
Kryostat: Leica CM 1900	Leica Microsystems, Wetzlar, Germany
Laser scanning microscope: Leica TCS SP	Leica Microsystems, Wetzlar, Germany
Metamorph Imaging software 7.0	Molecular Devices, Downingtown, PA, USA
Sigma Plot 8.0/ Sigma Stat 2.03	Systat Software, Erkrath, Germany

3.1.2 Reagents

Product:	**Company:**
2-Mercaptoethanol	Sigma-Aldrich Chemie, Munich, Germany
2-Propanol	Merck, Darmstadt, Germany
Acetone 99,8%	Merck, Darmstadt, Germany
Agarose	Molecular Probes, Leiden, The Netherlands
Amiloride	Sigma-Aldrich Chemie, Steinheim, Germany
Antibody diluent reagent solution	Zymed® Laboratories Inc., San Francisco, CA, USA
Aqua ad iniectabilia (H_2Odd)	Baxter, Unterschleißheim, Germany
Arginine	Sigma-Aldrich Chemie, Munich, Germany
Bovine serum albumine (BSA)	Sigma-Aldrich Chemie, Munich, Germany
Captopril	Sigma-Aldrich Chemie, Munich, Germany
DAPI	Linaris, Wertheim, Germany
DQ-casein	Molecular Probes, Leiden, The Netherlands
DQ-gelatine	Molecular Probes, Leiden, The Netherlands
Dulbecco‘s modified eagle medium (D-MEM)	Invitrogen, Karsruhe, Germany
Dulbecco’s phosphate buffered saline (PBS)	PAA Laboratories, Pasching, Austria
Enrofloxacin (Baytril®)	Bayer, Leverkusen, Germany
Eosin Y Disoldium salt	Sigma-Aldrich Chemie, Munich, Germany
Erythrocyte lysis buffer	Biolegend, San Diego, CA, USA
Ethanol	Riedel-de Haën Sigma-Aldrich, Seelsze, Germany
Fetal calf serum (FCS)	Invitrogen, Karsruhe, Germany

Formalin solution (PFA)	Carl Roth, Karlsruhe, Germany
Glycerol	Sigma-Aldrich Chemie, Munich, Germany
Glycine	Sigma-Aldrich Chemie, Munich, Germany
Hematoxylin solution	Merck, Darmstadt, Germany
Heparin	Ratiopharm, Ulm, Germany
Histofix 4 % (PFA)	Carl Roth, Karlsruhe, Germany
Hydrogen Peroxide 30% (H_2O_2)	Merck, Darmstadt, Germany
Lysine	Sigma-Aldrich Chemie, Munich, Germany
Methanol	Merck, Darmstadt, Germany
Non-immune goat serum 10%	Zymed® Laboratories Inc., San Francisco, CA, USA
Penicillin / Streptomycin	PAA Laboratories, Pasching, Austria
Phloxine B	Sigma-Aldrich Chemie, Munich, Germany
Plasminogen	From blood of healthy volunteers
Pluronic F-127 gel, 25% wt/vol	Sigma-Aldrich Chemie, Munich, Germany
RMPI medium 1640	Invitrogen, Karsruhe, Germany
Sodium acetate	Fluka Chemie, Buchs, Switzerland
Sodium chloride 0,9% (NaCl)	B. Braun, Melsungen, Germany
Sodium chloride solution	Baxter S.A., Lessines, Belgium
Sodium citrate	Carl Roth, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich Chemie, Munich, Germany
Trypan blue solution (0.4%)	Sigma-Aldrich Chemie, Munich, Germany
Tween 20	Sigma-Aldrich Chemie, Munich, Germany
Vectashield® mounting medium H 1000 for fluorescence	Vector Laboratories, Burlington, CA, USA
Xylocaine 2 %	AstraZeneca, Wedel, Germany

3.1.3 Surgical Instruments

Product:	**Company:**
Artery forceps BH111	Aesculap, Tuttlingen, Germany
Cautery	ZIK- Medizintechnik, Marktheidenfeld, Germany
Iris dissecting forceps OC021R	Aesculap, Tuttlingen, Germany
Iris dissecting forceps OC022R	Aesculap, Tuttlingen, Germany
Straight spring wire C-SF-15-20	William Cook Europe, Bjaeverskov, Denmark
Vannas style Eye scissors OC498R	Aesculap, Tuttlingen, Germany

3.1.4 Drugs used for anesthesia

Product:	**Company:**
Atropinsulfate-solution 0,5 mg/ml	Fresenius Kabi, Bad Homburg, Germany
Isoflurane	Baxter, Unterschleißheim, Germany
Ketamine 50 mg/ml	Inresa, Freiburg, Germany
Xylazine 2 % (Rompun ®)	Bayer, Leverkusen, Germany

3.1.5 Further materials

Product:	**Company:**
Butterfly perfusion set Micro Flo™, 0,5 × 20 mm (25 Gau)	Ind. Biomedica, Spa, Italy
Cover glass for slides	Menzel, Braunschweig, Germany
Dako® Pen	DakoCytomation, Glostrup, Denmark
Diamand® $D_{10/100/200}$ Certified Quality Tips 10µl//100µl/200µl	Gilson™ International B.V., Den Haag, The Netherlands
Ethilon® 6-0 Silk suture polyamid, not absorbable	Ethicon®, Johnson & Johnson, St.Stevens-Woluwe, Belgium
Ethilon® 7-0 Silk suture, polyamid, not absorbable	Ethicon®, Johnson & Johnson, St.Stevens-Woluwe, Belgium
Nylon sieve	BD Pharmingen, Franklin Lakes, NJ, USA
Fix-o-gum, Rubber-cement	Marabuwerke, Tamm, Germany
Parafilm®	American National Can™, Neenah, WI, USA
Pipettes 1000 µl / 200 µl / 100 µl / 10 µl	Eppendorf, Wesseling-Betzdorf, Germany
Safe Seal Microcentrifuge Tubes 0.65 ml/2 ml	Sorenson ™ Bioscience, Inc., Salt Lake City, UT, USA
Single-use syringe 1ml , Injekt F, Tuberkulin	B. Braun, Melsungen, Germany
Single-use syringe 5ml , Injekt Solo	B. Braun, Melsungen, Germany
Skin disinfection Softasept®	B. Braun, Melsungen, Germany
Slides Super Frost® Plus	Menzel, Braunschweig, Germany
Sterile needle, 20 Gau, 0,9 × 70 mm	Terumo® Euope, Leuven, Belgium
Sterile needle, 30 Gau, 0,3 × 13 mm	Terumo® Euope, Leuven, Belgium
Sterile needle, BD Microlance ™ 26 Gau, 0,45×13mm	BD Drogheda, Ireland
Tissue Tek® OCT ™ Compound	Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands

3.1.6 Antibodies and staining kits

Primary Antibodies

Antibody	Source	Isotype	Company
α-Smooth Muscle Actin	mouse	IgG monoclonal Cy3 conjugated	Sigma-Aldrich Chemie GmbH, Munich, Germany
Anti human FSAP (mAb1189)	mouse	IgG monoclonal	CSL Behring, Marburg, Germany
Anti human FSAP (mAb677)	mouse	IgG monoclonal	CSL Behring, Marburg, Germany
Anti mouse FSAP	rabbit	IgG polyclonal	CSL Behring, Marburg, Germany
CD31 (PECAM-1)	rat	IgG_{2a}, k	BD Pharmingen, Franklin Lakes, NJ, USA
CD41 (integrin α_{IIb} chain)	rat	IgG_1, k	BD Pharmingen, Franklin Lakes, NJ, USA
CD45	rat	IgG monoclonal	Serotec, Oxford, UK
CD68 (macrosialin)	rat	IgG monoclonal	Serotec, Oxford, UK
Control antibody	rabbit, rat, goat	various	Santa Cruz Biotechnology, Santa Cruz, CA, USA
eGFP	rabbit	IgG polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Fibrinogen	rabbit	IgG polyclonal	Dako, Glostrup, Denmark
MMP-2	goat	IgG polyclonal	R&D Systems, Mineapolis, MN, USA
MMP-9	goat	IgG polyclonal	R&D Systems, Mineapolis, MN, USA
MoMa-2	rat	IgG monoclonal	Serotec, Oxford, UK
uPA	rabbit	IgG polyclonal	American Diagnostica, Pfungstadt, Germany
vWF	rat	IgG polyclonal	Dako, Glostrup, Denmark

Secondary antibodies

Antibody	Source	Isotype	Company
Alexa Flour® 488 nm, green	goat	IgG polyclonal, anti-rabbit	Molecular Probes, Eugene, Oregon, USA
Alexa Flour® 546 nm, red	goat	IgG polyclonal, anti-rat	Molecular Probes, Eugene, Oregon, USA
Alexa Flour® 546 nm, red	donkey	IgG polyclonal, anti-goat	Molecular Probes, Eugene, Oregon, USA

Staining of proliferation

PCNA Staining Kit, Lot No.30476714	Zymed® Laboratories Inc., San Francisco, CA, USA

Staining of apoptosis

In Situ Cell Death Detection Kit C 2156793 TMR red	Roche Diagnostics GmbH, Mannheim, Germany

3.2 Methods

3.2.1 Mouse femoral artery injury model of neointimal hyperplasia

FSAP was isolated and provided by CSL Behring (Marburg, Germany). For all the experiments, FSAP was retained in a buffer (5mM sodium citrate, 50mM sodium chloride, 250mM arginine, and 200 mM lysine). Consequently, the controls were always performed by adding the same volume of the buffer.

Animals

Experiments were performed on adult male C57/BL6 mice purchased from Charles River (Sulzfeld, Germany). All procedures concerning animal experiments were in accordance with local ethical guidelines and had been approved by the institutional committee for animal research at Giessen University. The $uPA^{-/-}$ mice as well as the $uPAR^{-/-}$ mice with a C57/BL6 background were kindly provided by Dr. T. Bugge, NIH, Bethesda, Maryland, U.S.A.

Anesthesia

The mice were anesthetized by intramuscular injection of ~3 mg ketamine and ~2.5 µg xylazine (Rompun®) diluted in 0.9% sodium chloride solution into the right hind limb. Subsequently, 5 µg atropine was injected into the contralateral limb to antagonize the vagal effects of the anesthesia.

Surgery

Surgery was carried out using a dissecting microscope. Following anesthesia, the mice were fixed with tape and underwent transluminal mechanical injury of the left femoral artery by insertion of a straight spring wire (0.38 mm in diameter) for > 1 cm toward the iliac artery. This method was previously described by Sata *et al.* and modified by our group as described below[40]. In brief, the fur on the left hind limb was carefully removed with a scissor and afterwards the region of operation was disinfected. The skin was cut off from the distal end of

the leg in proximal direction for approximately 1 cm. The connective tissue around the femoral artery was removed with microsurgery forceps and the femoral nerve and femoral vein were carefully separated from the femoral artery by blunted dissection. This process was followed by preparation of the profunda femoris artery, where the arteriotomy was carried out to insert the spring wire. For this purpose, the femoral artery was looped proximally (Ligation I) with 6-0 silk suture for temporary vascular control during the procedure. In addition, the profunda femoris artery was ligated distally (Ligation II) with Ethilon 7-0 silk sutures. In preparation of the following dilatation, the ligations were stretched to prevent blood flow during the insertion of the wire. The exposed profunda femoris branch was dilated by topical application of xylocaine (2 %). Transverse arteriotomy was performed on the profunda femoris artery using Vannas style eye scissors (fig. 10).

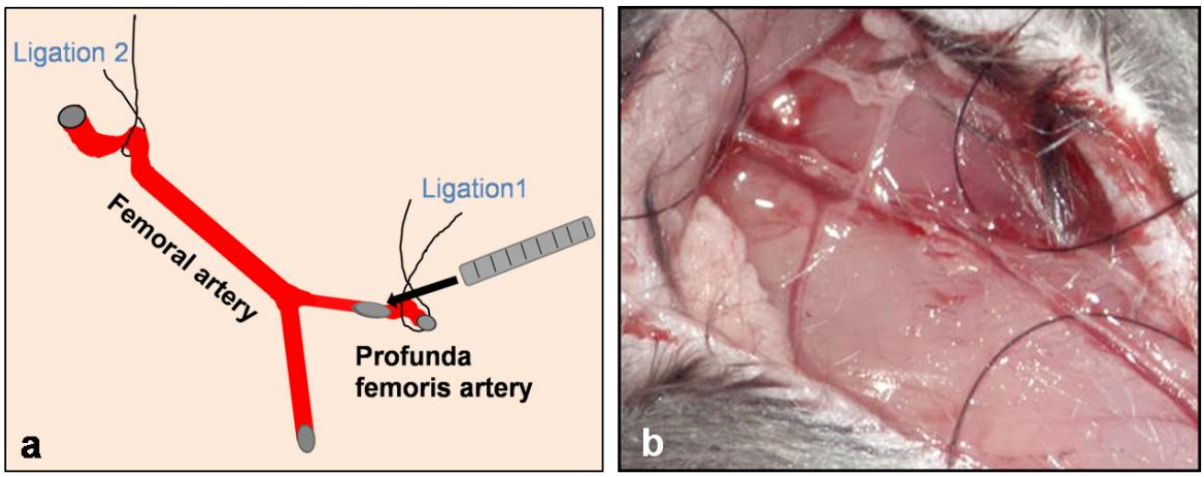

Figure 10. Wire induced endovascular injury of the femoral artery (1)

The left femoral artery was dissected, looped proximally with a 6-0 silk suture for temporary vascular control during the procedure (a, b). The profunda femoris branch was isolated and ligated distally with a 7-0 silk suture. Transverse arteriotomy was performed on the profunda femoris artery.

The straight spring wire was then carefully inserted into the profunda femoris branch and pushed forward to injure the femoral artery for approximately 1 cm. The wire was left in place for 1 min to denude and dilatate the artery (fig. 11).

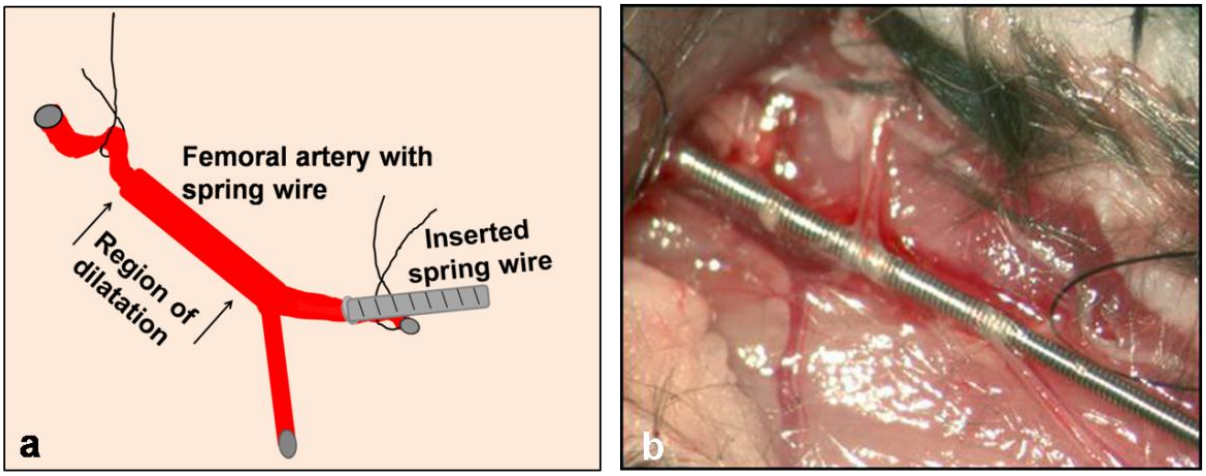

Figure 11. Wire induced endovascular injury of the femoral artery (2)

The straight spring wire was inserted and pushed forward into the femoral artery for more than 1 cm (a, b). The wire was left in place for 1 minute to denude and dilatate the artery.

The wire was removed, and the silk suture looped at the proximal portion of the profunda femoris artery was laced up to prevent blood loss from the arteriotomy. Blood flow of the femoral artery was restored by releasing the suture placed at the proximal side of the femoral artery.

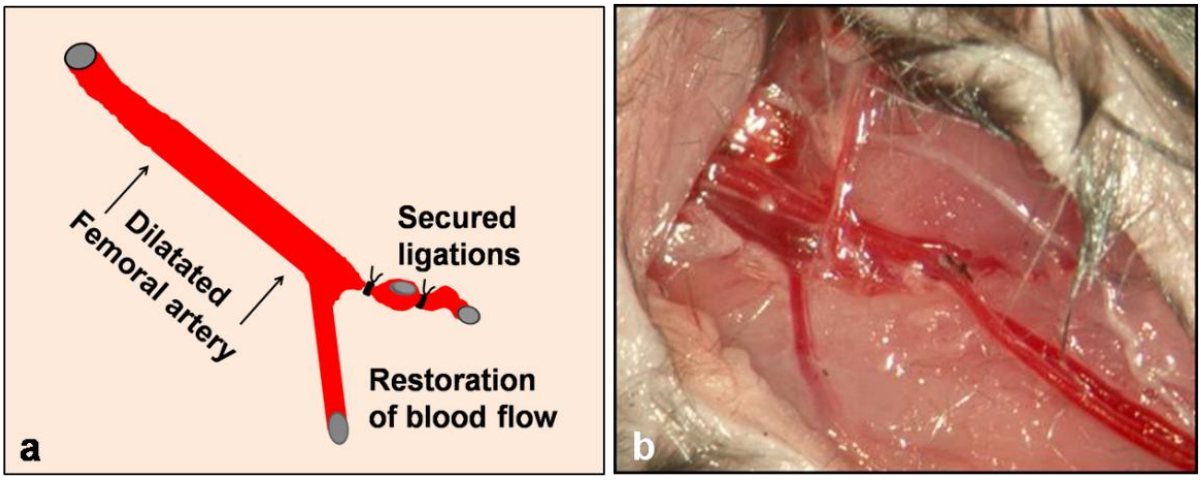

Figure 12. Wire induced endovascular injury of the femoral artery (3)

After removal of the wire, the proximal portion of the profunda femoris branch was tied off. Blood flow of the femoral artery was restored (a, b).

Immediately after dilatation, the artery was covered with 100 µl of a 25% thermosensitive pluronic F-127 gel containing FSAP (concentrations indicated in results) or buffer control. The fluid pluronic F-127 gel was administered at a temperature of 4°C and then solidified around the artery. The skin incision was closed with a 6-0 Prolene® silk suture.

Vessel harvesting

At the time points indicated, the mice were killed by an overdose of isoflurane. At death, the mice were perfused via the left ventricle with 2% PFA in PBS (pH 7.4). The femoral arteries were carefully excised, rinsed in PBS to remove remained blood and post-fixed in 2% PFA overnight at 4°C. The arteries prepared for zymography have not been fixed, respectively. Afterwards, the arteries were embedded in Tissue Tek® snap-frozen in liquid nitrogen and stored at -80°C until use. The whole arteries were systematically sectioned on a cryostat. The cross-sections (6 µm) were placed on poly-L-lysine coated slides for further staining.

3.2.2 Light microscopy staining and morphometry

Hematoxylin and Eosin (H & E) Staining

Hematoxylin and eosin staining was used on tissues for staining nuclei (blue, hematoxylin) and cytoplasm (red, eosin). Cryoslides were fixed in 4% PFA at room temperature for 10 min and rehydrated with PBS for 15 min. Hematoxylin staining was performed using Gill´s hematoxylin III for 6 sec and non-specific hematoxylin staining was removed by rinsing the slides with acetic acid. The slides were washed in running tap water for 10 min and then immersed in the eosin staining solution for 5 sec. The solution was prepared with 100 ml Eosin, 10ml Phloxin, 4 ml acetic acid, and filled up to the volume of 1 l with 95 % ethanol. This step was followed by dehydration in ascending alcohol solutions: 2 min 96% isopropyl alcohol supplemented with 0.6% acetic acid and twice with 100% isopropyl alcohol for 2 min each. The slides were mounted with Vectashield Mounting Medium and analyzed under the microscope.

Morphometric analysis

All sections were stained for hematoxylin and eosin before examination under a Leica DMRB microscope. Morphometric analysis was performed using Metamorph imaging software 7.0. The external elastic lamina, internal elastic lamina, and the lumen circumferences, as well as medial and neointimal area of six sections per artery were measured.

PCNA staining

To detect proliferating VSMC in neointimal tissue, mouse artery cross-sections were stained for the proliferating cell nuclear antigen (PCNA) by using Zymed's PCNA staining kit according to the manufacturer's instructions. In brief, tissue cross-sections were fixed in 4°C

PFA for 15 min, rehydrated in PBS for 10 min and then blocked with Blocking Solution (Reagent 1) for 10 min all at room temperature. The biotinylated mouse anti-PCNA primary antibody (Reagent 2) was applied for 60 min at room temperature to the sections. This step was followed by rinsing with PBS for 3x 2 min and application of the strepdavidine peroxidase (Reagent 3) for 10 min at room temperature. The slides were washed with PBS for 3x 2 min and the DAB chromogen mix was added to incubate for 5 min. The slides were counterstained with hematoxylin (Reagent 5) for 2 min and subsequently washed in running tap water and rinsed with H_2Odd until the sections turned blue. The slides were dehydrated in a graded series of alcohol, and cleared with xylene before covered with Histomount (Reagent 6) and a coverglass.

3.2.3 Immunohistochemistry

Immunohistochemistry

The following antibodies were used in the dilution indicated:

Antibody	**Dilution**	**Antibody**	**Dilution**
α-Smooth Muscle Actin	1:500	FSAP antibodies	1:100
CD31 (PECAM-1)	1:200	eGFP	1:200
CD41 (integrin αIIb chain)	1:200	MMP-2/9	1:100
CD45	1:200	MoMa-2	1:200
CD68 (macrosialin)	1:200	uPA	1:100
Fibrinogen	1:200	Von Willebrand Factor	1:100

Slides were fixed in 4% PFA for 15 min at room temperature, rehydrated in PBS for 15 min and blocked for 10 min with 10% normal goat serum. The primary antibodies were diluted in ready-to-use antibody diluent and incubated overnight at 4°C. Subsequently to rinsing with PBS for 3x 5 min, the slides were incubated with Cy5- or Cy3-coupled secondary antibodies diluted in antibody diluents (1:200) for 1 h at room temperature in a dark chamber. After incubation, the slides were rinsed again with PBS for 3x 5 min. The cross-sections were counterstained with DAPI dissolved in Vectashield® Mounting Medium. DAPI was used to identify the nuclei of the cells. In the case of anti-mouse FSAP antibodies using the N-terminal peptide as an antigen, an excess of free peptide was applied to test the specificity of the staining. Monoclonal antibodies to human FSAP and α-sma were labelled directly with Alexa 488 or Cy3, respectively. Negative controls were conducted by substituting the primary

antibody through an appropriate species and isotype matched control antibody. The cross sections were analyzed using fluorescent light microscopy.

TUNEL-staining

Staining for apoptotic cells was performed using Roches's *in situ* cell death detection kit. DNA fragmentation in apoptotic cells was detected by TdT-mediated dUTP-biotin nick end-labeling (TUNEL) according to the manufacturer's instructions. The slides were fixed in 4% PFA for 20 min at room temperature, rehydrated in PBS for 30 min and blocked with 3 % H_2O_2 in methanol for another 10 min. The slides were then incubated with a permeabilisation solution for 2 min at 2°C, which contained 0.1 % Triton X-100 and 0.1 % sodium citrate dissolved in PBS to permeabilize the cell membrane. The slides were rinsed with PBS for 3x 2 min. The cross-sections were then incubated with the TUNEL reaction mixture for 1 h in a dark and humidified chamber at 37°C and additionally covered with parafilm. After incubation, the slides were rinsed with PBS for 3x 2 min, counterstained with DAPI in mounting medium and analyzed using fluorescence microscopy.

Microscopical analysis

Tissue samples were analyzed using immunofluorescence imaging with deconvolution analysis of high resolution *z*-axis image stacks. This technique was performed in a subset of sections by using a blind 3D deconvolution algorithm (Autoquant Deblur 9.3; Autoquant Imaging). Furthermore, we performed laser scanning microscopy in a subset of sections.

3.2.4 Zymography

In situ zymography of MMP2/ MMP-9 and uPA:

In situ zymography was performed on frozen and unfixed sections (6 μm) as described before[89]. In brief, sections were overlaid with a mixture of agarose and dye quenched (DQ)-gelatin, and the fluorescence was measured immediately to detect the background staining. After an incubation time of 24h, fluorescence images were captured again, and gelatinolytic activity (MMP-2 and MMP-9) was determined as an increase in fluorescence over background. Presence of the Zn^{2+} ion chelator, captopril, inhibited all gelatinolytic activity. To measure the activity of plasminogen activators by casein zymography, the overlay consisted of DQ-casein, plasminogen and agarose. The PA in the tissue activated plasminogen to plasmin, which in turn cleaved the DQ-casein. Fluorescence reached its maximum after 4

hours. Amiloride, an urokinase inhibitor, reduced plasminogen activation, thus indicating that active uPA was present in the tissue section. As another control, $uPA^{-/-}$ mice showed a strongly reduced cleavage of DQ-casein. Immunolocalization of the respective proteases was performed with an anti-uPA antibody and anti-MMP-2 as well as anti-MMP-9 antibodies according to the methods described before.

3.2.5 Irradiation and bone marrow transplantation (BMTx)

Irradiation

Male C57/BL6 mice purchased from Charles River were irradiated at the Institute for radiotherapeutics with either 10.5 Gy or 9.5 Gy of gamma-radiation (as indicated in results). The aim of the irradiation was to ablate the BM of the mice.

BMTx

At one day after irradiation, the mice were transplanted with BM from enhanced green fluorescence protein positive ($eGFP^+$) mice. The $eGFP^+$ mice were kindly provided by Dr. R. Voswinckel, MPI, Bad Nauheim. For this purpose, the $eGFP^+$ mice were euthanized and the BM of the femur as well as the tibia was rinsed out with RMPI Medium 1640 containing 1 % fetal calve serum (FCS) and penicillin/streptomycin. This suspension was filtered through a nylon sieve (20µm), and an erythrolysis was performed using erythrocyte lysis buffer. After counting the cells using a hemocytometer (Neubauer's counting chamber), we injected ~3 x 10^6 bone marrow cells into the tail vein of each irradiated mouse (fig. 13). Enrofloxacin (Baytril®) was administered to the drinking water for 2 weeks after transplantation. Wire induced dilatation of the mouse femoral artery was performed at 8 weeks after BMTx as described before, and vessels were harvested at the dates indicated in results.

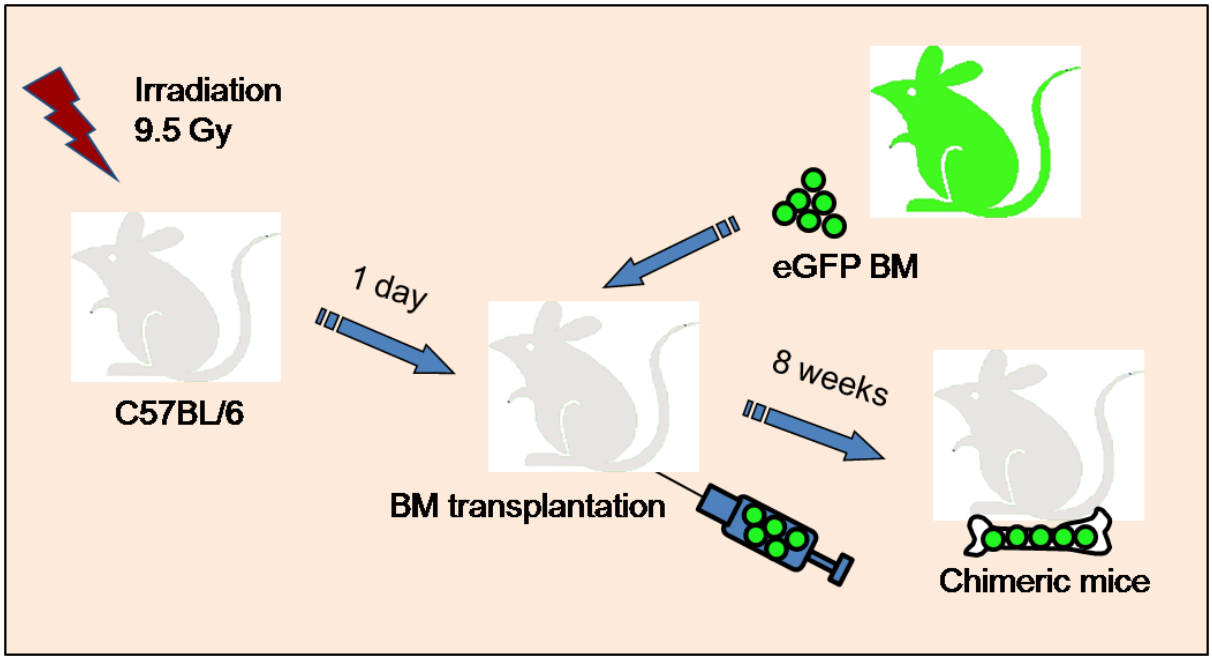

Figure 13. Irradiation and BMTx of eGFP$^+$ cells

The mice were irradiated with 9.5 Gy and subsequently rescued by transplanting eGFP$^+$ BM cells into the tail vein.

Flow cytometry

At 8 weeks after transplantation, flow cytometry analysis of blood samples was performed on five randomly chosen chimeric mice. The mice were killed at 2 h after an i.p. injection of heparin. Approximately 200µl of whole blood was lysed in erythrocyte lysis buffer. The fraction of eGFP$^+$ leucocytes in peripheral blood was measured in a forward-side scatter gate. In addition, we analyzed peripheral blood leucocytes at the time points of harvesting the arteries.

3.2.6 Western Blotting

Western blotting analysis of human FSAP released from pluronic F-127 gel:

Single chain FSAP (10 µg/ml) was added to 25% (wt/vol) pluronic F-127 gel at 4°C. The mixture was then heated and allowed to solidify for 30 min at 37°C. Thereafter, an equal volume of serum-free D-MEM was added to the solidified gel. At the indicated times (5 sec to 46 h), the buffer as well as the solidified gel were separated and mixed with SDS sampled buffer and boiled. For the analysis of FSAP, the samples were either non-reduced or reduced with 2-mercaptoethanol (10%, vol/vol). For Western blot analysis of human FSAP, a

combination of two monoclonal mouse antibodies (mAb677 against the light-chain and mAb1189 against the heavy-chain) was used as described before[78].

3.2.7 Statistical analysis

Data were stored and analyzed on personal computers using Excel 2003 (Microsoft) and Sigma Plot 8.0 with Sigma Stat 2.03. Data between the study groups were analyzed by ANOVA followed by pairwise comparison with Fisher's least significance test. All calculations were made with the Statgraphics plus statistical package.

4. Results

4.1 Physiological expression of FSAP in tissue extracts

FSAP is predominantly produced as an inactive single-chain zymogen in the liver and then secreted into the circulation[66]. In Western blot experiments, an anti-mouse FSAP antibody could detect single-chain FSAP and its degradation products in mouse plasma with concentrations probably comparable to the levels observed in human plasma (data provided by Dr. Kanse).

Immunofluorescence analysis with a polyclonal anti-mouse FSAP antibody using the N-terminal peptide as an antigen showed an intensive staining for FSAP in the liver of the mice. To show the specificity of the staining, an exogenously added excess of free peptide was used as a control and revealed a negative staining. In the media of native vessels, only a faint scattered staining of FSAP was observed. In the developing neointima of dilatated arteries, the FSAP staining was analyzed at 7 days and 21 days after injury. At the early time point, the staining was not enhanced, and leucocytes adhering to the disrupted endothelium did virtually not express FSAP. At 21 days after dilatation, however, the staining pattern of FSAP was generally enhanced (fig. 14). Interestingly, the staining of mouse FSAP was similar to that of macrophages, which were particularly located in the medial layer. In a co-staining for mouse FSAP and CD 68 (marker for macrophages), this observation could be confirmed.

No FSAP mRNA transcripts were detected in resident cells of femoral or carotid arteries, but high levels were found in the liver and also in monocyte-derived macrophages (qPCR data provided by Dr. Kanse). Hence, FSAP in the vasculature is not produced locally but is derived by diffusing from the circulation and by infiltrating monocyte-derived macrophages.

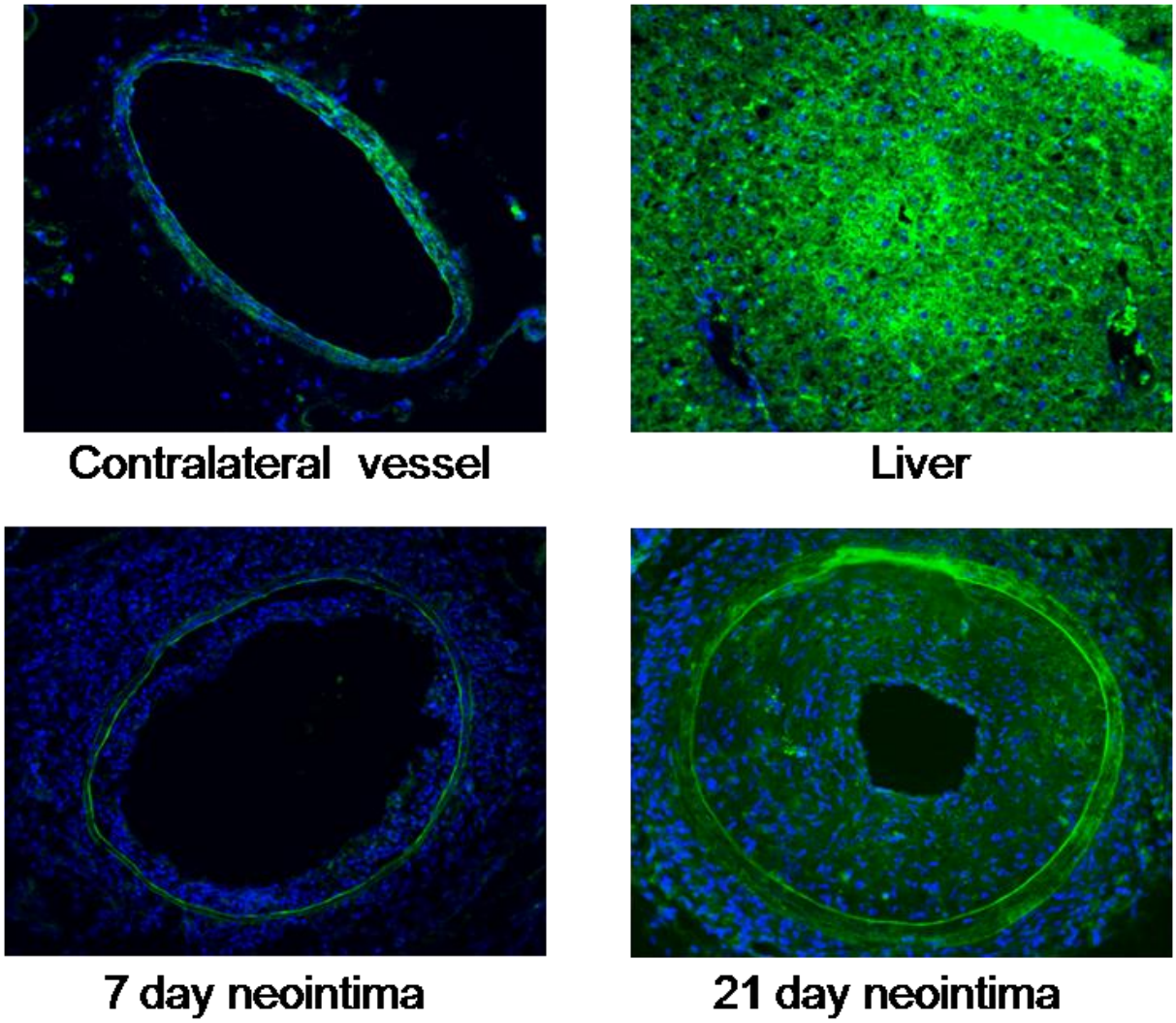

Figure 14. Localization of endogenous mouse FSAP

Immunofluorescence analysis was performed with a polyclonal anti-mouse FSAP antibody on uninjured vessels, dilatated vessels after 7 and 21 days, and the liver as a positive control. Only a faint staining was observed in the vessel wall. At 21 days after dilatation, there was generally more staining, especially in the macrophage rich medial layer.

4.2 Release of FSAP from Pluronic F-127 gel

Human FSAP used in the experiments was provided by CSL Behring (Marburg, Germany), and the specific effects of human FSAP on mouse VSMC have been well demonstrated[78]. There was no recombinant mouse FSAP available at the times of the experiments, so that exogenous human FSAP was applied to the denuded mouse arteries. The release of FSAP from the thermosensitive pluronic F-127 gel was investigated *in vitro* and *in vivo*.

In the *in vitro* experiments, there was a slow sustained release of intact FSAP from the pluronic F-127 gel over a period of 24 h (fig. 15 A). Furthermore, 100 µl of pluronic F-127

gel containing FSAP was applied to a mouse artery and the amount of FSAP present in the gel was analyzed at various timepoints. When applied abluminally to mouse arteries *in vivo*, FSAP was released from the gel within 1h. There was a complete resorption of FSAP from the gel at later timepoints, thus indicating that FSAP indeed diffused into the artery (fig. 15 B).

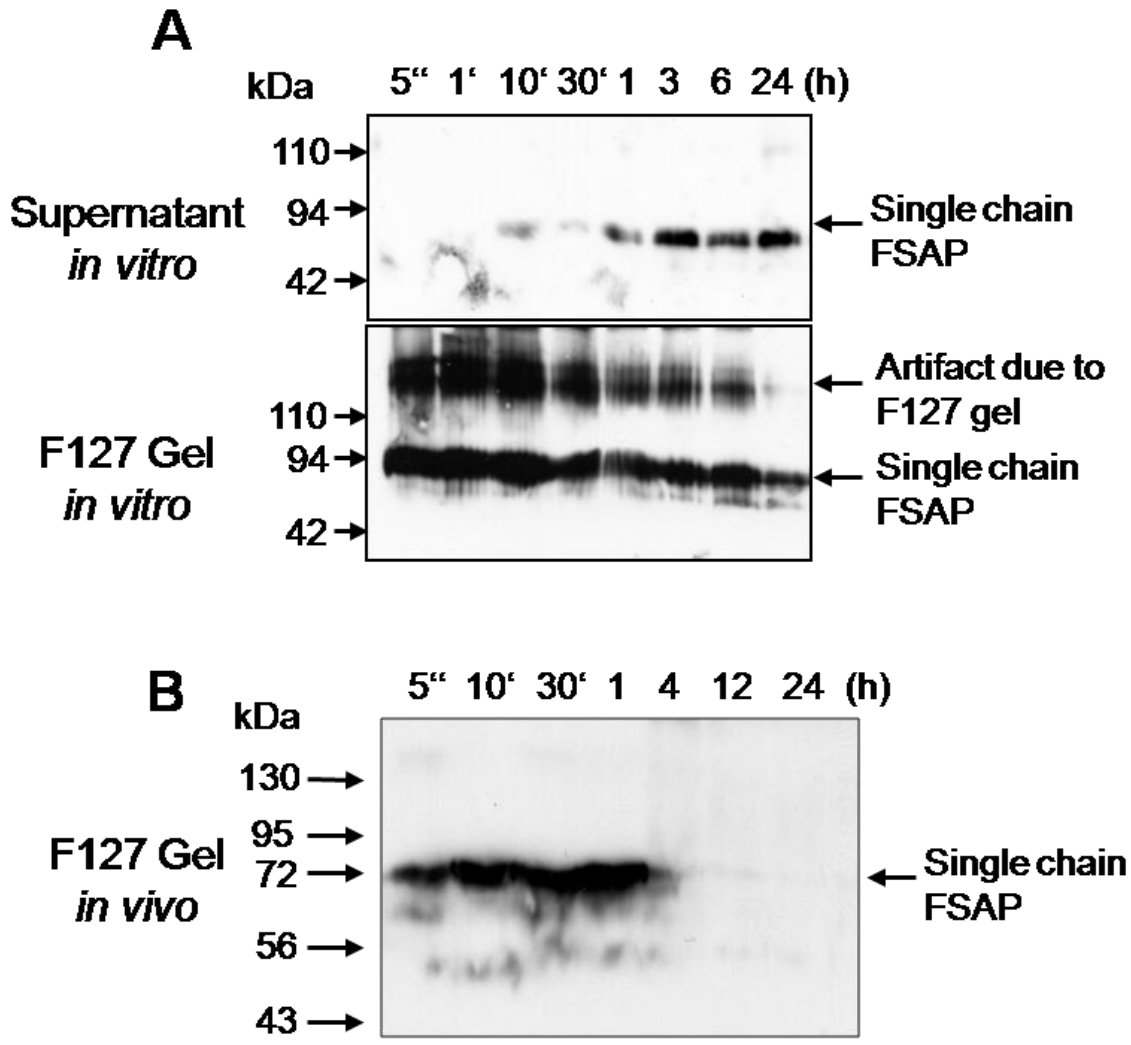

Figure 15. FSAP release from pluronic F-127 gel in vitro

FSAP was added to pluronic F-127 gel in PBS at 4°C, and then the mixture was shifted to 37°C and covered with a buffer. The supernatants and the pluronic gels were separated at the indicated time points and analyzed for human FSAP using a mixture of two monoclonal antibodies. Since pluronic F-127 gel influences the migration properties of proteins in SDS-PAGE, it gives rise to artifacts (A). For an *in vivo* experiment, we added 1 µg of FSAP to 100 µl of pluronic F-127 gel and applied the mixture to a mouse artery. At the indicated time points, the pluronic gel was recovered and analyzed for the presence of human FSAP (B).

To verify the presence of exogenous human FSAP in the vascular wall, we performed immunofluorescence staining of FSAP in dilatated arteries. Application of human FSAP immediately after injury markedly increased immunoreactivity in vessels after 12 and 48 h. After 21 days, the staining dominated the circumference of the artery. The vessels treated with buffer showed virtually no immunoreactivity (fig. 16). The monoclonal anti-human FSAP antibody used for these experiments (mAb677) did neither cross-react with mouse FSAP in plasma samples, nor with tissue extracts of mouse aorta or liver in Western blotting experiments (data provided by PD Dr. Kanse). Hence, FSAP was constantly released from the gel, and it was present in the artery at an early and crucial phase of neointima formation.

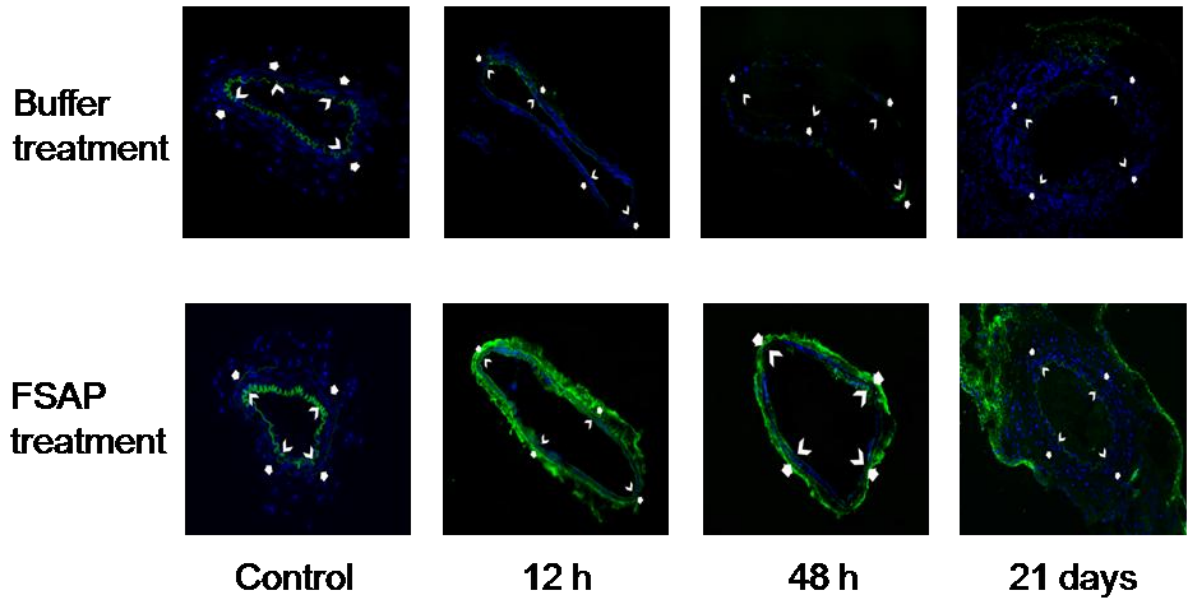

Figure 16. Uptake of FSAP into the vessel wall

FSAP diffused from the pluronic F-127 gel into the vessel wall and could be detected 12h and 48h after dilatation with an anti-human FSAP antibody (mAb677). At 21 days after dilatation, the exogenously administered FSAP was predominantly present in the surrounding of the vessel.

4.3 FSAP attenuates neointima formation

The influence of FSAP on neointimal thickening was determined at 3 weeks after dilatation. Wild-type (WT)-FSAP was administered in concentrations of 1µg, 0.5 µg and 0.2 µg in pluronic F-127 gel to the denuded artery. Since the *in vitro* inhibitory effect of FSAP on VSMC was neutralized after protease inactivation, we compared these different concentrations to 1 µg of the active-site inhibited Phe-Pro-Arg-chloromethyketone (PPACK-) FSAP, 1µg Marburg I (MI-) FSAP, and a buffer control group. In contrast to WT-FSAP, MI-FSAP has reduced proteolytic activity towards its direct chromogenic substrate, and it is also

a weaker activator of pro-uPA. Active-site inhibited PPACK-FSAP did not possess any enzymatic activity but was otherwise equivalent to normal FSAP[79] (data provided by Dr. Kanse).

Each group of dilatated mice consisted of 6 mice, and 6 different sections of a defined range of the artery were analyzed for the morphometry of the neointima. WT-FSAP application led to a significant and dose dependent decrease in intima/ media ratio. The maximal inhibition (~70%) was achieved by application 0.5-1µg WT-FSAP to the denuded artery compared to the buffer control group. Accordingly to the *in vitro* data, MI-FSAP showed only a marginal and non-significant reduction, and PPACK-FSAP had no effect on neointima formation (fig. 17 A, B). There were corresponding alterations in the lumen area and the intimal area in the different treatment groups but no changes in the medial area.

As a further control, we inhibited the *in vivo* effects of the exogenously applied WT-FSAP by additionally administering aprotinin to the pluronic F-127 gel, in order to neutralize the proteolytic activity of FSAP. Aprotinin (1.4 µg/mouse) by itself reduced neointima/ media ratio, but in combination with FSAP it reversed the inhibitory effect of FSAP. This reversal was not complete and the original vehicle-control levels were not attained indicating a general inhibitory effect of aprotinin on neointima formation independently of the neutralization of FSAP.

Since FSAP was applied locally to the injured artery, the hypothesis is that FSAP diffuses into the artery and thereby mediates its inhibitory effect rather than having a more systemic mode of action. This was further investigated by applying FSAP to the contralateral uninjured artery and not directly to the denuded artery. Distant application of FSAP on the contralateral artery was ineffective in reducing neointima/media ratio.

Hence, the exogenously administered FSAP attenuates neointima formation in a dose dependent manner and promotes its inhibitory effect by diffusing locally into the artery rather than having a more systemic mode of action. This hypothesis is also in accordance with the immunofluorescence analysis of human FSAP released from pluronic F-127 gel *in vivo*.

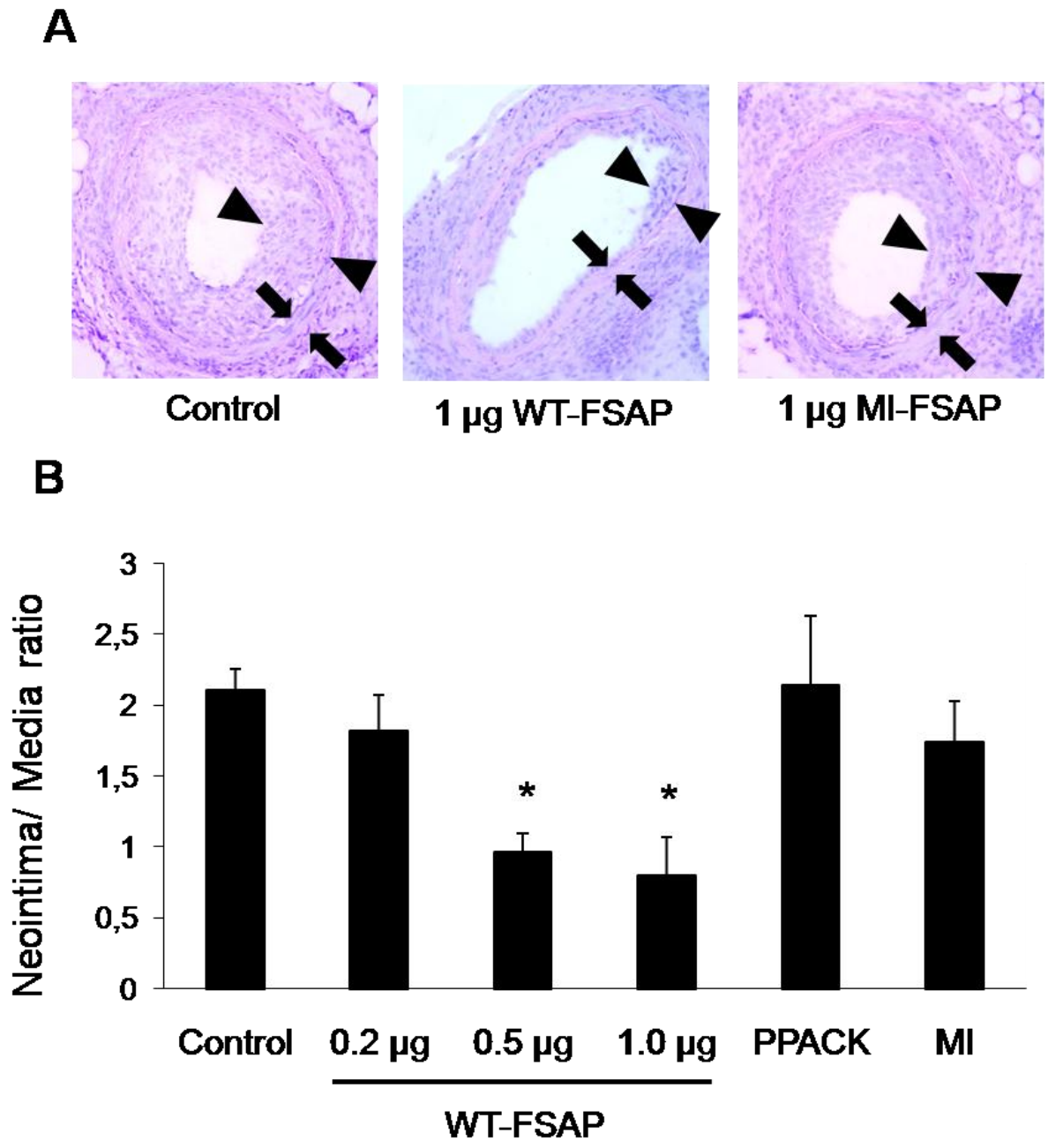

Figure 17. WT-FSAP inhibits neointima formation in a dose dependent manner

Immediately after injury, 100 µl of pluronic F-127 gel was applied to the artery containing either buffer, human WT-FSAP (0.2, 0.5, 1 µg), PPACK-FSAP, or MI-FSAP (1.0 µg). In the hematoxylin and eosin (H&E)-stained sections, arrowheads indicate the neointimal lesion and arrows mark the medial layer (A). The neointima/ media ratio was calculated (mean ± SD, n = 6 mice) with Metamorph 7.0 software (B).

4.4 FSAP inhibits VSMC proliferation in the developing neointima

FSAP inhibited PDGF-BB derived proliferation of VSMC *in vitro* and was shown in the first experiments to reduce neointima formation *in vivo*[78]. In regard to these results, the

immunofluorescence staining of α-sma as a marker for VSMC was significantly reduced in WT-FSAP-treated vessels compared with the control groups. The extent of staining correlated with the size of neointimal thickening, indicating that WT-FSAP inhibits the accumulation of VSMC in the neointima in a dose dependent manner. The staining of α-sma in the media showed virtually no difference among the treatment groups (fig. 18 A).

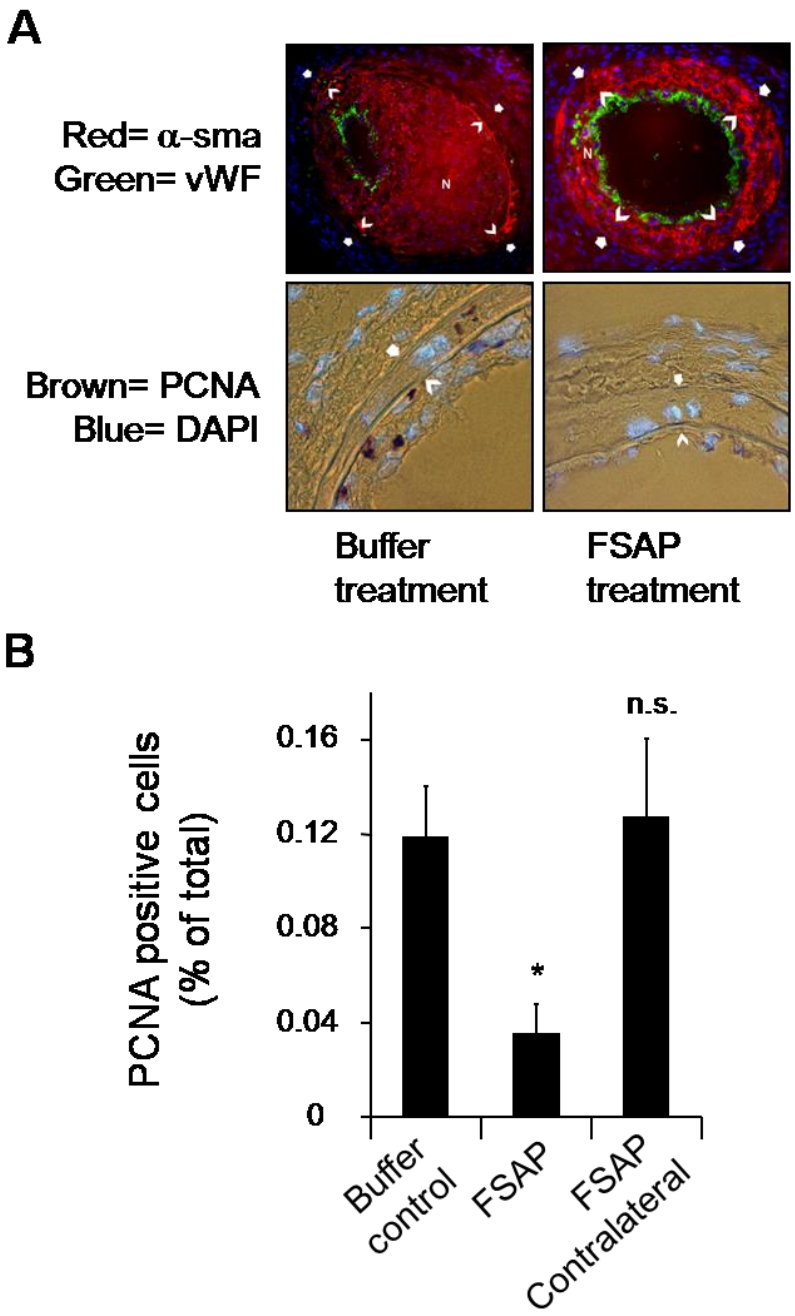

Figure 18. FSAP inhibits proliferation of VSMC

The antibody detecting α-sma was directly conjugated with Cy-3 (red), whereas vWF was stained with a FITC-conjugated secondary antibody to detect endothelial cells. PCNA was stained brown with a biotin-streptavidin peroxidase kit (A) and numbers of proliferating cells have been calculated (B).

Furthermore, the number of proliferating cell nuclear antigen (PCNA; or Ki76)-positive cells in the neointima and in the media was systematically analyzed. At 3 weeks after dilatation, the number of PCNA-positive cells in the neointima was reduced by ~70% after WT-FSAP application compared to the control group (fig. 18 B). Hence, proteolytic active FSAP inhibits VSMC proliferation *in vivo* and thus reduces neointima formation.

4.5 Effects of FSAP on apoptosis of VSMC, accumulation of monocytes/ macrophages and re-endothelialization

The neointima formation is not only dependent on the proliferation/ migration of medial VSMC but also on the rate of apoptotic VSMC after injury and triggering mechanisms, e.g. infiltration of monocytes/ macrophages to the sites of the denuded vessel[90, 91]. Most of the medial VSMC undergo apoptosis immediately after dilatation of the artery. To examine the effects of FSAP on injury induced apoptosis, the number of TUNEL-positive cells in WT-FSAP treated vessels (1µg) and in the buffer control group was analyzed at 24 h after dilatation. Application of FSAP to the denuded artery did not modulate the rate of apoptosis of VSMC, as determined by the percentage of TUNEL-positive cells in relation to all remaining cells (DAPI) within the media. Hence, FSAP does not influence this early step of neointima formation (fig. 19).

The recruitment of monocytes/ macrophages was also not altered after FSAP treatment. At 3 weeks after dilatation, macrophages could predominantly be detected in the medial layer and in the surrounding of the artery (CD68 staining). There was virtually no difference after treatment with WT-FSAP compared to the control groups. The neoinitmal lesion, however, was also interspersed with macrophages. Since the size of the neointima was reduced in FSAP treated vessels, the absolute number of macrophages in the neointimal layer was reduced, respectively.

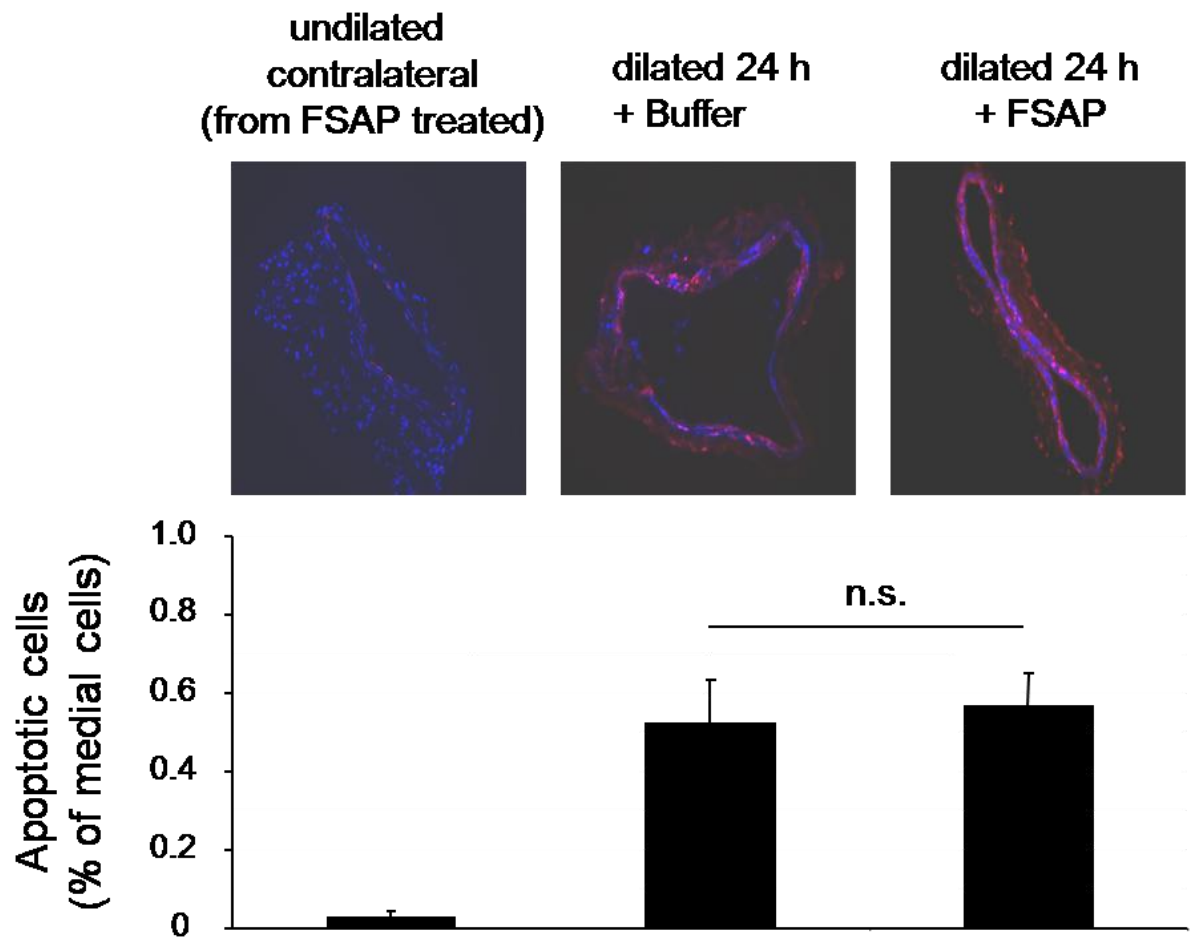

Figure 19. Influence of FSAP on apoptosis of VSMC after dilatation

After injury, 100 µl of pluronic F-127 was applied to each artery either containing human WT-FSAP (1 µg) or buffer. After 24 h, the vessels were stained with an apoptosis detection kit based on TUNEL staining (Roche Diagnostics). The percentage of apoptotic cells was defined as TUNEL-positive cells in relation to all remaining cells (DAPI) within the media (mean ± SD, *n* = 6 mice).

Since FSAP is involved in both the coagulation and the fibrinolysis system, we stained the dilatated arteries for fibrin deposition. Interestingly, the reduced neointima in FSAP treated mice featured a higher density in fibrin staining (fig. 20 A).

After wire induced endoluminal rupture of the endothelium, there was virtually no difference in re-endothelialization with or without FSAP treatment at 3 weeks after dilatation. In all analyzed groups, the immunofluorescence staining for platelet/ endothelial cell adhesion molecule (PECAM)-1 (or CD31) as well as vWF revealed an intact endothelial layer at 3 weeks after injury (fig. 20 B).

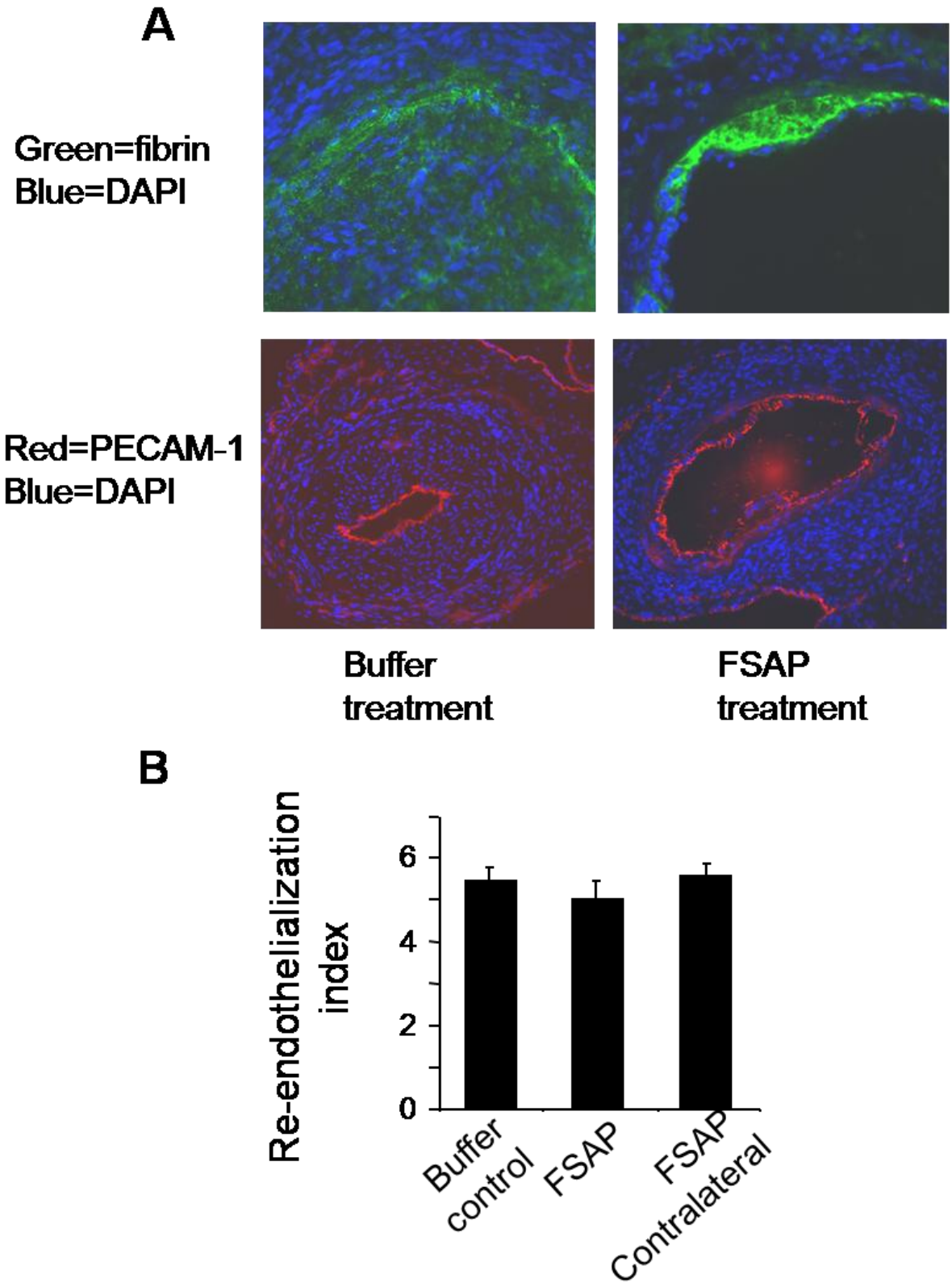

Figure 20. Fibrin deposition and Re-endothelialization

Immunofluorescence staining for fibrin and PECAM-1 was performed after FSAP treatment or buffer treatment alone. Re-endothelialization was determined by estimating the lumen coverage on a scale of 0-6 (0, no coverage; 6, complete coverage).

4.6 Effect of FSAP on the plasminogen activation system and the matrix-metalloproteinases (MMP) in vascular remodeling

Since FSAP has the ability to activate pro-uPA, the inhibition of proliferation and neointima formation in the vessel wall is possibly related to modulation of endogenous plasmin or MMP activity. In EC and VSMC, a down-regulation of uPA was observed in the presence of FSAP (our unpublished observations). To examine the alterations of the pericellular proteolysis balance by FSAP in the process of vascular remodeling, we performed *in situ* zymography and investigated the effect of FSAP treatment on the plasminogen activation system and on MMP-2 and MMP-9 (MMP-2/9) activation. The vessels were dilatated, and either 1 µg of WT-FSAP, 1 µg of MI-FSAP, or the control buffer was administered to the pluronic F-127 gel. The arteries were harvested at 2 days (n=2), 14 days (n=3) and 21 days (n=3) after injury without being perfused and fixed with PFA, respectively. The activity of MMP-2/9 was measured by gelatin zymography, and this activity could be specifically inhibited by the Zn^{2+} chelator, captopril. In the course of neointima formation, the MMP-2/9 activity markedly increased. At 14 days after injury, the MMP-2/9 activity was predominantly located in the medial layer, whereas after 21 days the developing neointima was more prominent in MMP-2/9 activity. WT-FSAP treated arteries exhibited increased gelatinolytic activity after 14 and 21 days (fig. 21 A), and there was a concomitant increase in MMP-2 and in MMP-9 immunostaining compared to MI-FSAP or buffer control (fig. 21 B).

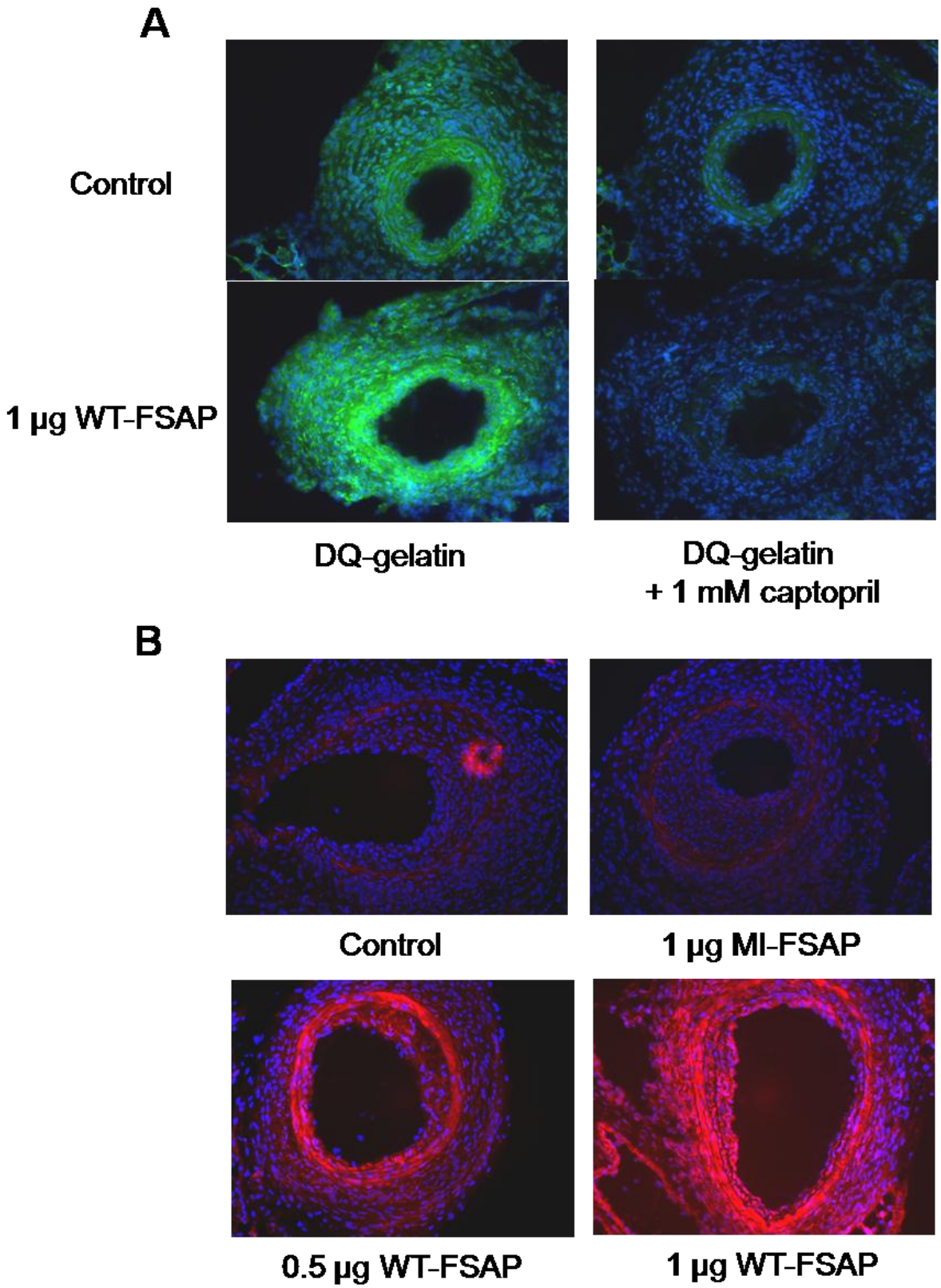

Figure 21. Effects of FSAP on the pericellular proteolysis system (1)

An *in situ* gelatin-zymography was performed to determine the activity of MMP-2/9 activity with or without additional application of captopril at 2 weeks after injury (A). Furthermore, immunohistochemistry was performed to confirm the zymography results. Staining for MMP-9 is shown at 2 weeks after injury for different treatment groups (B).

In situ zymography with casein as a substrate in the presence of plasminogen was used to quantify the endogenous plasminogen activation capacity. This activity was inhibited in the presence of the uPA inhibitor, amiloride. The uPA activity was rather low in non dilatated vessels, and it increased in all treatment groups in the course of neointima formation. The activity of uPA also dominated the medial area at 14 days after injury and was then notably detected in the neointima at 21 days after injury. In conformity with the up-regulation of the pericellular proteolysis system in vessels treated only with the control buffer, a concomitant staining of PCNA revealed high proliferative indices of SMC in the media at 14 days and in the neointimal lesion at 21 days of neointima formation. Interestingly, the uPA activity was decreased in WT-FSAP treated vessels at 14 and 21 days after injury (fig. 22). Furthermore, there was a parallel decrease in uPA immunostaining in accordance with the results obtained from the zymography experiments.

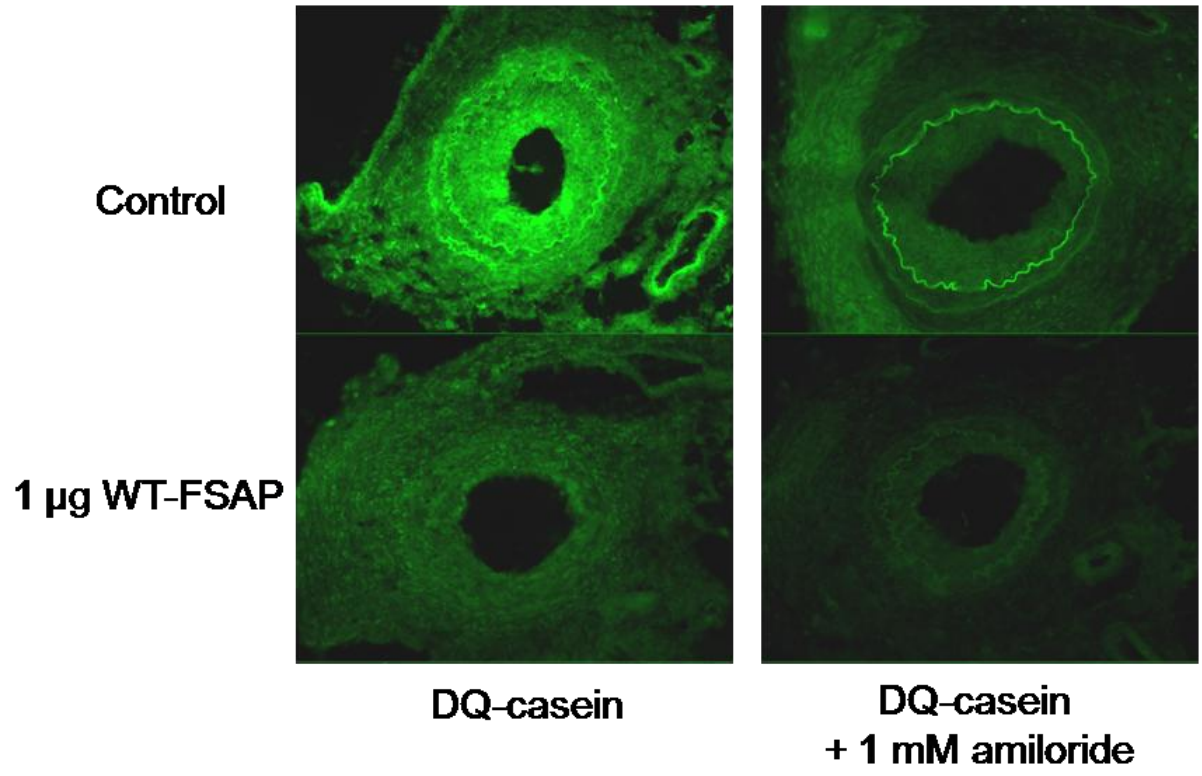

Figure 22. Effects of FSAP on the pericellular proteolysis system (2)

An *in situ* casein-zymography was performed to determine the endogenous plasminogen activators at 3 weeks after injury. After additional application of the uPA inhibitor, amiloride, the activation capacity of endogenous plasminogen activators was reduced, thus indicating the importance for uPA in endovascular plasminogen activation.

4.7 Neoinitma formation in uPA$^{-/-}$ mice

The neointima formation in uPA$^{-/-}$ mice has been characterized before in models of ferric chloride, perivascular electric injury or carotid artery ligation. All these experiments demonstrated the importance of the uPA-system for the proliferation of VSMC in vascular remodeling. The uPA$^{-/-}$ mice did not develop a cell-rich neointima and media, but the lumen of the arteries, however, was narrowed with acellular thrombotic material at the sites of injury[62, 63].

To further investigate the effects of FSAP on uPA activity, age matched uPA$^{-/-}$ mice were dilatated with application of WT-FSAP or control buffer to the pluronic F-127 gel. The arteries of the mice (n=6) were analyzed 3 weeks after injury and compared to the control group of WT-mice. The size of the neointima was not reduced, but it consisted mainly of acellular thrombotic material. In contrast, the size of the media was attenuated, and only a few VSMC could be detected in the neointima or in the media of the artery (fig. 23 A, B). Consequently, the number of PCNA (or Ki76) positive cells was also reduced in uPA$^{-/-}$ mice compared to WT-mice. FSAP application did not influence neointima formation in uPA$^{-/-}$ mice, and it did not change the size or the organization of the acellular thrombotic material. The proliferation and migration of VSMC in neointima formation have already been substantially inhibited by the knock out of the uPA-system, so that an additional effect of FSAP could not be attained in uPA$^{-/-}$ mice. However, since FSAP led to a down-regulation of the endovascular uPA activity over time, the analog reduction of VSMC proliferation in FSAP treated WT-mice and uPA$^{-/-}$ mice suggests the mode of action of FSAP to be dependent on the uPA-system. Hence, the markedly reduced cellularity in uPA$^{-/-}$ mice shows the importance of the uPA-system in arterial remodeling, and the down-regulation of uPA in wild type mice after treatment with WT-FSAP partly reflects the effects on arterial remodeling in uPA$^{-/-}$ mice.

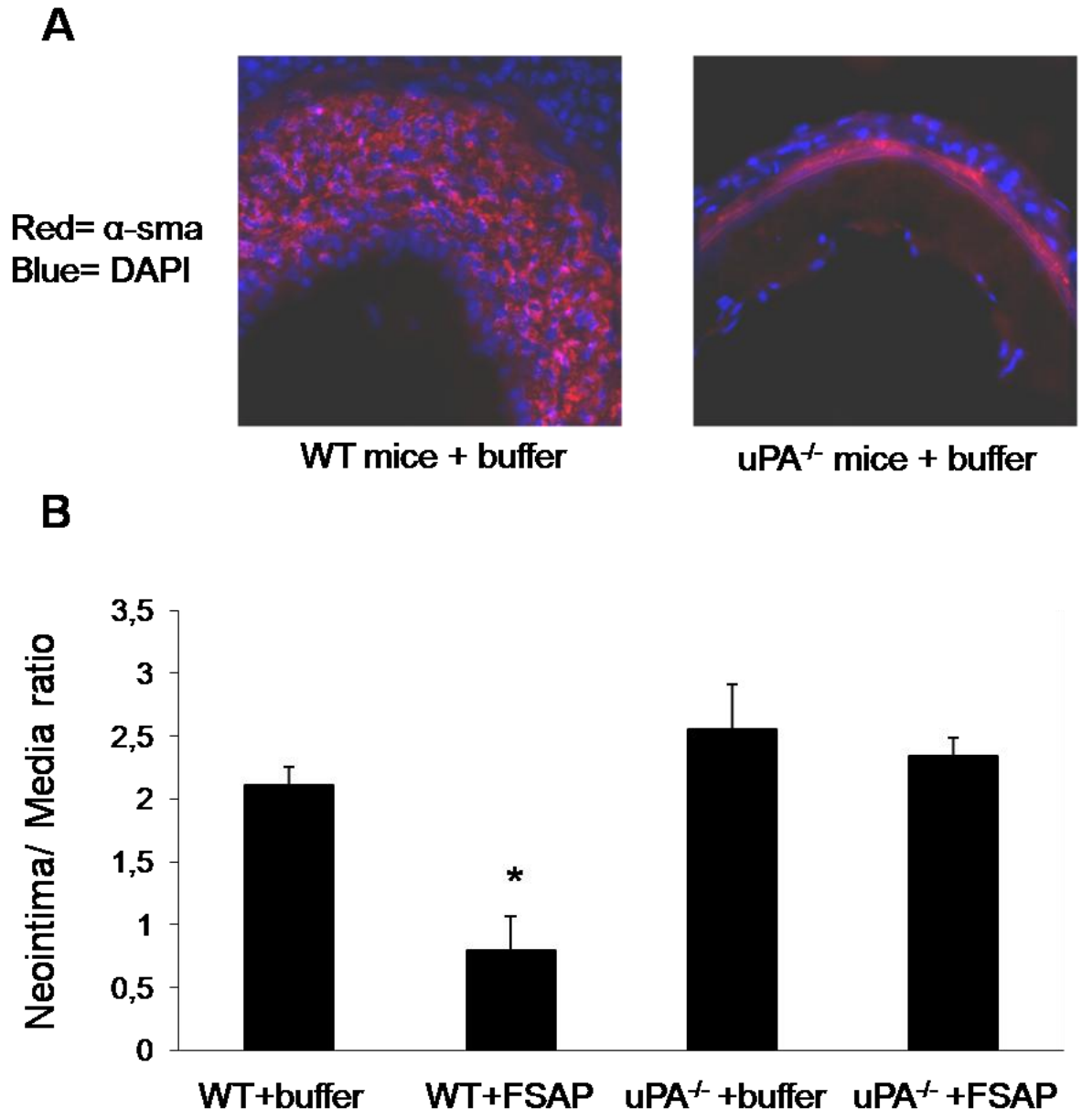

Figure 23. Neointima formation in uPA$^{-/-}$ mice

In uPA$^{-/-}$ mice, the neointima was not composed of VSMC but thrombotic and acellular material, as determined by staining for α-sma (A). The neointima/ media ratio was calculated (mean ± SD, n = 6 mice) (B).

4.8 Effect of FSAP on the transdifferentiation of bone marrow derived progenitor cells (BMPC) into VSMC in neointima formation

Recent studies claim that circulating bone marrow derived progenitor cells play an important role in neointima formation and atherosclerosis by transdifferentiating into VSMC[42, 92]. Moreover, FSAP reduced the *in vitro* differentiation of c-kit/ stem cell antigen (sca)-1 positive cells into α-sma expressing cells from 82,1% to 19,1% (20ng/ml PDGF-BB and 20ng/ml

SDF-1α, unpublished observations by Dr. Kanse). Since we could show a strong inhibitory effect of FSAP on VSMC *in vivo*, it was examined, whether this effect might be due to an impairment of BM-derived cell transdifferentiation.

C57BL/6 mice were irradiated with 10.5 Gy and subsequently rescued with bone marrow cells from eGFP$^+$ mice. FACS analysis of chimeric mice revealed that 85-95% of circulating mononuclear cells expressed eGFP at 8 weeks after BMTx.

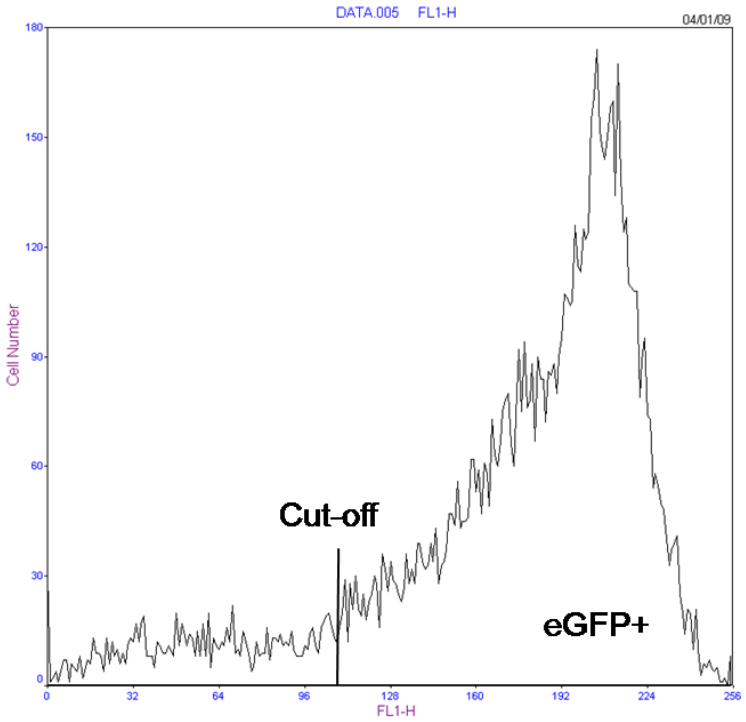

Figure 24. Flow cytometry analysis of chimeric mice

The fraction of eGFP$^+$ leucocytes in peripheral blood was measured in a forward-side scatter gate at 8 weeks after BMTx.

The mice were then dilatated, and the pluronic F-127 gel was applied to the denuded artery containing either 1 µg of WT-FSAP or the control buffer. The arteries were harvested at 4 weeks after injury (n=6) and subjected to histological analysis. The later time point for the extraction of the vessels was chosen because of the deleterious effects of the irradiation on neointima formation described in the literature[93, 94].

The eGFP$^+$ cells were analyzed for the expression of α-sma using immunofluorescence imaging and deconvolution analysis of high resolution *z*-axis image stacks as well as confocal microscopy. Surprisingly, following careful analysis throughout the complete lesion range, the expression of α-sma in eGFP$^+$ cells occurred to be a very rare event in the neointimal lesions of both control arteries and arteries treated with FSAP (fig. 25 A).

After irradiation with 10.5 Gy and subsequent BMTx, the size of the neointima at 4 weeks after injury was reduced compared to the neointimal lesion at 3 weeks after dilatation in non pre-treated mice (neointima/ media ratio 1.90±0.17 vs. 2.11±0.15). Direct application of 1 µg FSAP to the denuded artery led to a reduction in neointimal thickening in BM-transplanted mice by 69% (fig. 25 B). The neointima of the control group consisted of regular organized VSMC, as determent by α-sma staining (fig. 25 A). The $eGFP^+$ cells in these lesions were predominantly identified as monocytes/ macrophages. In contrast, FSAP treatment led to a neointima that nearly completely lacked VSMC and consisted mainly of $eGFP^+$ leucocytes (CD45 staining) and thrombocytes (CD41 staining) as the principal components. According to the results in the non-irradiated mice, the density of fibrin staining was enhanced after application of FSAP, as well.

In the neointima of FSAP-treated vessels, a 43% higher percentage of $eGFP^+$ cells was detected due to an almost complete lack of resident VSMC after 4 weeks. The absolute number of $eGFP^+$ cells, however, was not significantly different with or without FSAP treatment. Positive cells for CD68 targeting macrophages were predominantly detected in the medial layer and in the surrounding of the vessel. There was virtually no difference after FSAP treatment except for the alignment of the cells in the unequal neointima. Interestingly, the CD31 staining for endothelial cells revealed a missing re-endotheliazation in the FSAP-treated vessels at 4 weeks after injury in the irradiated and BM-transplanted mice (fig. 25 A). In the buffer treated arteries, the endothelium lining the neointima was well developed at 4 weeks after dilatation. The lack of VSMC and EC in FSAP-treated arteries suggests an impaired stability of the neointima, but no differences in thrombus formation were detected compared to buffer treated vessels.

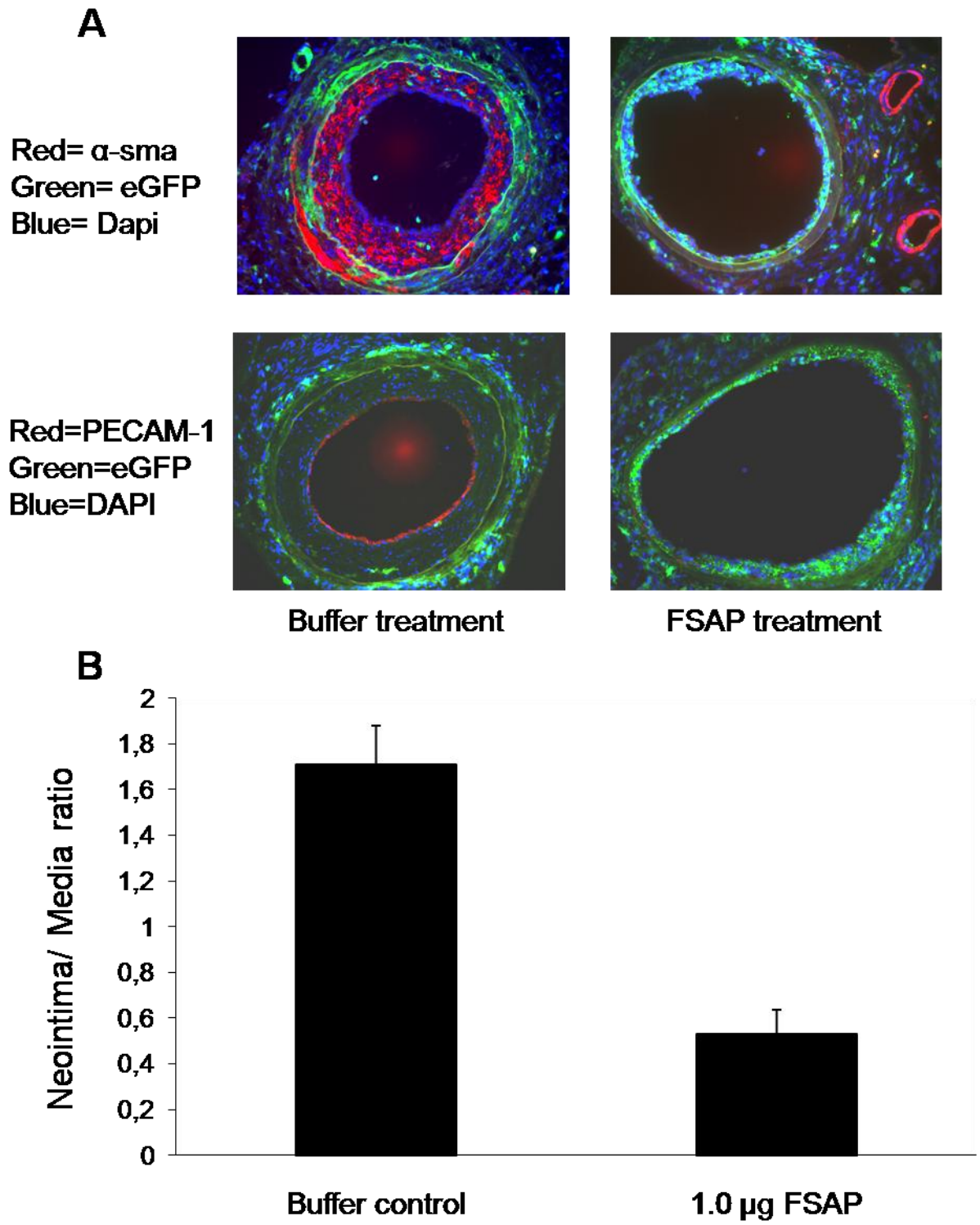

Figure 25. Effect of FSAP in irradiated and BM transplanted mice

After irradiation and BMTx, application of FSAP to the denuded artery led to a reduction of the neointima/ media ratio. Importantly, the neointimal lesion nearly completely lacked VSMC and EC in FSAP treated arteries (A). The neointima/ media ratio was significantly reduced and corresponded to the results obtained in non-irradiated mice (mean ± SD, n = 6 mice) (B).

Hence, FSAP inhibits proliferation as well as migration of resident VSMC. Since the absolute numbers of $eGFP^+$ cells did not differ significantly, FSAP does not imply any *in vivo* effects on adhesion of BM-derived cells to the injured artery. Or, if it does, these mechanisms cannot be detected in the model of neoinitma formation used in the experiments. There was no *in vivo* transdifferentiation of BMPC at all, so that a possible inhibition of FSAP on cells derived from the circulation could be ruled out.

4.9 Time course analysis of BMPC transdifferentiation in vascular remodeling

On the basis of the results obtained from the first BMTx experiments, the aim was to further elucidate the role of circulating BMPC into VSMC during vascular remodeling. Since only little information is available about the time points of accumulation, differentiation and the long term contribution of BMPC compared to media derived VSMC, a time course experiment was performed. After irradiation with 9.5 Gy, mice were reconstituted with bone marrow cells from $eGFP^+$ mice according to the first experiment. FACS analysis again revealed that 85-95% of circulating mononuclear cells expressed eGFP at 8 weeks after transplantation. Wire induced dilatation of the mouse femoral artery was performed, and vessels were harvested after 3 days, 1, 2, 3, 4, 6 and 16 weeks (n=8 animals per time point).
The size of the neointima increased over a time period of 4 weeks (neoinitma/ media ratio of 2.13 + 0.26) and slightly decreased thereafter until 16 weeks of vascular remodeling (neointima/ media ratio of 1.78 + 0.19) (fig. 26 A, B). At 3 days after dilatation, the endothelium was disrupted and the denuded/injured luminal surface was lined with thrombocytes, as determined by CD41 staining. In the first weeks, the neointima was predominantly composed by accumulating $eGFP^+$ leucocytes to the injury site. In response to wire induced injury, a substantial fraction of medial VSMC underwent apoptosis directly after dilatation. However, VSMC could steadily be detected in the medial layer at all time points after dilatation. At 2 weeks after injury, a peak in the recruitment of $eGFP^+$ leucocytes was observed, and at 3 weeks, the first α-sma expressing cells started to accumulate in the neointima (fig. 26 A).

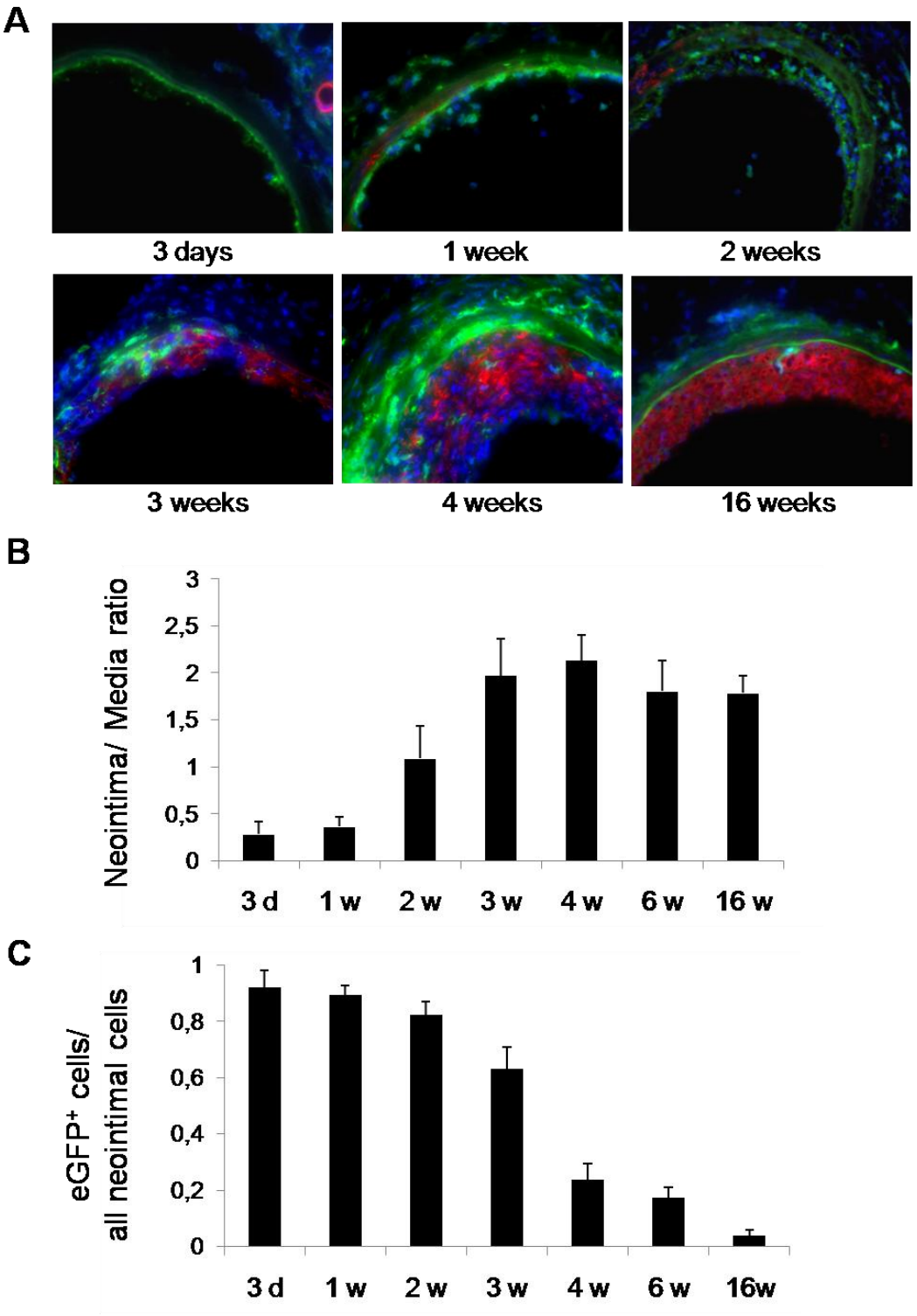

Figure 26. Time course experiment on the origin of VSMC

Thrombocytes adhered to the disrupted endothelial layer at 3 days after wire-induced injury followed by an accumulation of leucocytes. At 3 weeks after injury, the first resident and eGFP negative cells started to migrate toward the neointimal lesion and reached a maximum in size after 4 weeks. At 16 weeks after injury, hardly any $eGFP^+$ cells could be detected in the neoinitma (A). The size of the neointima increased over a time period of 4 weeks and slightly decreased thereafter until 16 weeks of vascular remodeling (B). The absolute number of $eGFP^+$ cells in the neointima as well as the $eGFP^+$ cells/ all neontimal cells ratio continuously declined at later time points (C).

Especially at 2 and 3 weeks after injury, the proliferative indices of VSMC in the medial layer of the dilatated arteries were very high, as determined by PCNA staining. Furthermore, the medial VSMC proximal of the dilatated area showed very high proliferative indices, as well. The complete lesion range was carefully analyzed using deconvolution analysis of *z*-axis image stacks and high resolution confocal microscopy. In accordance with the previous results, the expression of α-sma in GFP^+ cells of the vessel wall occurred to be a very rare event.

The absolute number of $eGFP^+$ cells in the neointima as well as the $eGFP^+$ cells/ all neontimal cells ratio continuously declined at later time points after injury due to both high apoptotic indices of $eGFP^+$ cells and increasing numbers of eGFP negative VSMC in the neointima. Consequently, only very few BM-derived cells could be detected in the neointima at later time points (fig. 26 C). According to the decline of $eGFP^+$ cells, there was no long term contribution of any transdifferentiated BM-derived VSMC to arterial remodeling (fig. 26 A). The $eGFP^+$ cells in the vessel wall were predominantly identified as macrophages.

Hence, these data provide evidence that transdifferentiation of BMPC into VSMC lineages seems to be a relatively rare event. Moreover, the contribution of BMPC to the cellular compartment of the neointimal lesion is limited to a temporary time period of the inflammatory response to the vascular injury. This time course experiment clearly indicates that the effect of FSAP on neontimal thickening is derived from the inhibition of resident VSMC and does not have any effect on BMPC transdifferentiation.

5. Discussion

The MI-SNP of FSAP is associated with late complications of carotid stenosis and is a general risk factor for atherosclerosis[4, 5]. Since this polymorphism is present in ~5% of the European population, the effect of FSAP on vascular pathophysiology is an interesting and important question considering the large number of patients suffering from atherosclerosis related illnesses. For the studies presented here, the *in vitro* experiments characterized MI-FSAP as a protease with a weaker enzymatic activity compared to WT-FSAP. MI-FSAP is also a weaker inhibitor of PDGF-BB derived cell proliferation and cell migration[79]. In the wire induced dilatation model of neointima formation, WT-FSAP inhibits neointima formation but MI-FSAP does not. From these results it can be inferred that the function of endogenous FSAP is to inhibit VSMC cell proliferation and that in patients with the MI-genotype this inhibition is lost, thus leading to an odds ratio of 6.63 in the risk profile of advanced atherogenesis in the Bruneck study[4].

On the other hand, VSMC do have beneficial functions in atherosclerotic plaques, as well[29]. They form the fibrous cap and protect the lipid core from rupture and cardiovascular events. Indeed, FSAP was shown to be present in instable human plaques, so that the inhibition of PDGF-BB may lead to a thinning of the VSMC in the fibrous cap. Additionally, FSAP expression and FSAP mRNA could be detected in macrophages, and a co-localization with uPA was observed[75]. The down-regulation of uPA and the up-regulation of MMP-2/9 by FSAP in our *in situ* zymography experiments are absolutely concordant with the clinical observations of elevated vulnerability of the plaques expressing FSAP. In FSAP treated vessels of irradiated and BM-transplanted mice, the neointima was predominantly composed of thrombocytes and leucocytes. The neointimal lesion was further characterized by an almost complete lack of VSMC and an impaired re-endothelialization probably due to the additional effects of FSAP and irradiation on the resident cells. Since the control mice developed a cell rich neointima after BMTx and dilatation, the local application of FSAP destabilized the neointima by reducing the cellularity of the lesion. We identified the cleavage of PDGF-BB, the down-regulation of uPA as well as the up-regulation of MMP as the key processes in FSAP activity.

Hence, further powerful clinical trials are warranted to shed more light on the clinical features of the MI-SNP with respect to cardiovascular events and rates of restenosis. A detailed analysis of the morphology of the plaques in MI-carriers is also indispensable for discussing a

possible treatment of these patients in cooperation with the results from basic research experiments and the ongoing clinical studies.

In regard to the potential therapeutic effects of FSAP on proliferating VSMC in neointima formation, the use of FSAP on drug-eluting stents could be beneficial to prevent restenosis. In non-irradiated arteries, FSAP did not inhibit re-endothelialization at 3 weeks after injury despite a strong inhibition of VSMC, which is an important condition to prevent in-stent thrombosis after PTA and stenting. However, because of the incomplete endothelial layer after irradiation and FSAP treatment, we cannot definitely exclude a possible inhibition of re-endothelialization by FSAP. Furthermore, FSAP has been shown to influence the proliferation of endothelial cells *in vitro*[95], and an inhibitory effect of FSAP on angiogenesis was detected in a murine tumor model (our unpublished observations). In conclusion, the effects of FSAP on EC need further experimental efforts to be clarified.

Since administration of FSAP to the contralateral vessel did not mediate a systemic effect on the neointimal lesion in our *in vivo* model, a systemic effect of FSAP derived from a stent on atherosclerotic lesions apart from the dilatated artery seems unlikely. Accordingly, FSAP as an endogenous protease can rapidly be inhibited by serine protease inhibitors after dissolving from a stent to the circulation[96].

5.1 Inhibition of proliferating VSMC via platelet derived growth factor (PDGF)-BB cleavage

In our *in vitro* experiments, FSAP inhibited the PDGF-BB stimulated DNA synthesis in VSMC and concomitantly blocked the PDGF-BB-dependent phosphorylation of p42/p44 MAPK (ERK) and tyrosine phosphorylation of other proteins. These effects have not been observed by MI-FSAP and have been linked to the enzymatic activity of WT-FSAP[79].

The analysis of dilatated arteries clearly showed an inhibitory effect of FSAP on VSMC proliferation and migration. The application of FSAP led to a 70% reduction in neointima formation and to a significant decrease in the number of PCNA (or Ki76) positive cells at multiple time points. Hence, the *in vitro* effects of FSAP on PDGF-BB inhibition can probably be directly transferred to the situation *in vivo*.

A number of investigations have concluded that PDGF-BB inhibition at early time points of neointima formation plays a key role to inhibit neointimal growth[80, 97]. These observations from various animal models are concordant with the *in vitro* finding that only PDGF-BB and no other growth factors have been essentially inhibited by FSAP. Lately, drug-eluting

balloons have been introduced into clinical practice, because application of anti-proliferative drugs at such early time points has been shown to potently inhibit the rates of restenosis[98]. Adhesion of thrombocytes and leucocytes to the disrupted endothelial layer along with the release of PDGF-BB are known to be crucial steps in the early phase of neointima formation[11, 99]. These pathophysiological features could also be demonstrated in our experiments. Since there was no difference in the accumulation of leucocytes to the ECM of the disrupted endothelial layer, the specific cleavage of PDGF-BB by WT-FSAP but not MI-FSAP had a more important effect than a possible alteration of adhesion molecules in leucocytes. However, there are preliminary data showing a possible regulation of adhesion molecules by FSAP (our unpublished observations), but at least with the model of neointima formation we could not detect any significant differences *in vivo*. Hence, specific cleavage of PDGF-BB is one of the major mechanisms by which FSAP is able to reduce cell proliferation and migration in the neointima.

5.2 FSAP influences the proteolytic system in the vascular wall

WT-FSAP and MI-FSAP differ in their ability to activate pro-uPA[100]. In cell culture experiments, there was a down-regulation of uPA in EC and in VSMC after incubation with WT-FSAP but not MI-FSAP (our unpublished observations). The *in situ* zymography of dilatated vessels exactly confirmed this effect *in vivo*. At 14 and 21 days after injury, the uPA activity was reduced in WT-FSAP treated vessels. Indeed, the importance of the uPA-system for proliferating VSMC has been described in various animal models[62, 63]. In our own experiments of wire induced injury, the $uPA^{-/-}$ mice did not develop a regular neointima composed of VSMC but a lesion of acellular thrombotic material. These results indicate that uPA is essential for maintaining the cellularity and, possibly, the stability of the plaques, both by preventing excessive fibrin accumulation at the sites of injury and by facilitating cell migration and invasion. The neointima of dilatated and FSAP treated arteries after irradiation and BMTx did not consist of VSMC but of leucocytes and thrombocytes. It featured both a high density in fibrin staining and an impaired re-endothelialization. The morphometry of these arteries was similar to that in $uPA^{-/-}$ mice, which almost completely lacked VSMC in the neointima. According to the acellular material forming the neointima in $uPA^{-/-}$ mice, the reduced uPA activity in FSAP treated vessels could, at least in part, account for the instable appearance of the neointima in irradiated and transplanted mice as compared to the irradiated mice of the control group. Thus, the inhibitory effects of FSAP on neointima formation may

be explained by a down-regulation of uPA in addition to the specific cleavage of PDGF-BB. Evidently, the knock out of the uPA-system markedly inhibited the proliferation and migration of VSMC per se, so that it was not possible to provide evidence for any effects of FSAP in $uPA^{-/-}$ mice.

In regard to the results from the *in vivo* experiments, it is not surprising that FSAP has been co-localized with uPA predominantly in unstable plaques[75]. Furthermore, advanced atherosclerotic lesions in humans show enhanced uPA inactivation and turnover[65]. Because of this uPA down-regulation, which is possibly induced by FSAP, the layer of VSMC forming the fibrous cap is thinned out and makes the plaque susceptible for rupture. Hence, the clinical observations perfectly fit with the results obtained from the *in vivo* experiments presented here.

Indeed, neointima formation is associated with increased activity of plasminogen activators and MMP[32]. FSAP decreases plasminogen activation potential but increases MMP activity. However, the latter observation is actually not consistent with an inhibition of neointima formation. MMP-2 and MMP-9 have been believed to crucially influence vascular remodeling, but other reports have relativized their importance. Filippov *et al.* provide evidence that membrane type (MT)-1 MMP (MMP-14) is the key player of the MMP-family in neointima formation and that MMP-2/9 do not significantly influence this process[101]. Consequently, the data from the literature on MMP activity in vascular remodeling are controversy, and the definite role of FSAP in this interplay is very difficult to evaluate. Nevertheless, it is clear that FSAP alters the balance of the proteolytic systems in vascular remodeling. Furthermore, the activation of MMP-2/9 by FSAP matches with the clinical finding of FSAP immunostaining in instable atherosclerotic plaques. The elevated MMP activation digests the ECM components and therefore attenuates the stability of the plaque and renders it more susceptible for rupture[32].

5.3 FSAP does not affect bone marrow derived progenitor cell (BMPC) transdifferentiation during neointima formation

In recent *in vivo* studies, BMPC were claimed to be the origin of more than 60% of the neointimal cells[42]. That was already reason enough to investigate the effect of FSAP on transdifferentiation of BMPC into VSMC. Additionally, FSAP was found to substantially reduce the *in vitro* transdifferentiation of BMPC (our unpublished observations). Surprisingly, the expression of α-sma in $eGFP^{+}$ cells occurred to be a very rare event in both FSAP treated

arteries and buffer treated arteries. We performed further experiments to verify that BM derived VSMC do not contribute to the cellularity of neointimal lesions, and the results will be discussed below.

The lack of VSMC in the neointimal lesion and the impaired re-endothelialization were probably due to the additive effects of FSAP and irradiation on VSMC. Especially the impaired re-endothelialization should be taken into account for a possible clinical use of FSAP on drug-eluting stents, since this might increase the risk of in-stent-thrombosis after PTA. In contrast, the neointimal lesion in non-irradiated arteries consisted of VSMC and we could also detect an adequate re-endothelialization by staining for CD31 and vWF. Nevertheless, the effects of FSAP on irradiated vessels have always been compared to control vessels of identically pre-treated mice. Because of the regular cellularity of the irradiated and buffer treated vessels, we thus refer the lack of VSMC and the delayed re-endothelialization to the inhibition of resident vascular cells by FSAP.

However, irradiation causes many deleterious effects on recipient animals. In many clinical studies on brachytherapy for preventing restenosis after PTA, irradiation was shown to reduce viability and proliferation of host cells[94]. However, the effects of a single shot irradiation at 8 weeks before induction of neointima formation in mice have not been systematically studied so far, and this was also not one of the primary aims of this study. Since we used different irradiation doses in our experimental setting, we can conclude that irradiation attenuates neointima formation in a dose dependent manner. In comparing the neointima formation in mice after irradiation and BMTx to non-pretreated mice, a delay of this process probably due to the irradiation could be confirmed. In contrast, control mice irradiated with 11.5 Gy hardly developed any neointimal lesion at 4 weeks after dilatation at all.

5.4 Contribution of BMPC transdifferentiation to vascular remodeling

To further investigate the role of BMPC in the process of neointima formation, a time course experiment confirmed that the transdifferentiation of BMPC into VSMC and EC appears to be a very rare event. Furthermore, there was no long term contribution of BMPC after the inflammatory response to the vascular injury had resolved, and most of the $eGFP^+$ cells in the neointima were identified to be macrophages. Recent studies have clearly demonstrated that BM-derived cells accumulate to the injured artery[99]. However, a transdifferentiation of BMPC into highly differentiated VSMC remains speculative, because these cells were only positive for α-sma, but not for more specific VSMC markers like calponine or vimentine. Since

macrophages can express α-sma under some circumstances, as well, we investigated the long term contribution of BM derived cells in vascular remodeling[102]. Importantly, the number of $eGFP^+$ cells in the neointima continuously declined during vascular remodeling, so that these cells do not imply a definite contribution to the cellular mass of the neointima over time. Moreover, we only find very low rates of transdifferentiated BMPC at earlier time points after wire induced injury. These results are in contrast to other publications, which claim to detect more than 60 % of eGFP and α-sma double positive cells after wire-induced injury[42]. To explain these controversial data, we have to face methodological limitations of many studies in the past. It is of critical importance that the use of unfixed tissue and a lack of high resolution microscopy do not provide convincing evidence for the process of transdifferentiation[103]. Indeed, when we abstained from immediate fixation with 4 % PFA after killing the mice, the tracer molecule diffused all over the tissue and could no longer be specifically detected in the $eGFP^+$ cells. Interestingly, a recent report by Bentzon *et al.* could not confirm the suggested transdifferentiation of BM-derived cells into VSMC in a model of collar induced atherosclerosis. The authors did not find a single VSMC of donor BM origin using fixed tissue and high resolution microscopy, thus providing excellent evidence that highly differentiated VSMC in atherosclerotic lesions originate entirely from the local vessel wall. However, it is claimed that the amount of transdifferentiated BMPC might be dependent on the severity of the injury. Indeed, a wire induced dilatation causes a distinctive inflammatory response and can account for false positive results by an overlap of GFP^+ cells and local derived VSMC. This artifact will be dependent on the number of infiltrating cells and thus on the severity of the injury. Moreover, the infiltrating macrophages can also temporarily express α-sma[102]. Consequently, these "differentiated SMC-like macrophages", as these cells were called in a recent editorial by Drs. Iwata and Sata, lose their relevance in the course of time. At 16 weeks after wire induced injury, the highly differentiated VSMC are exclusively derived from pre-existing local cells, and their "contractile" phenotype very much corresponds to the VSMC in a non dilatated artery.

This conclusion is also important for the clinical problem of restenosis after balloon induced dilatation of an occluded artery. The claimed BM-origin of VSMC in arterial remodeling would shift therapeutic attempts towards an inhibition of BMPC transdifferentiation in clinical burdens, such as in-stent-restenosis, venous graft failure, or transplant atherosclerosis. Although monocytes/ macrophages play an important role in neointima formation, our data provide evidence that transdifferentiation of BMPC into VSMC seems to be a very rare event.

5.5 Inhibition of proliferating VSMC and plaque stability: Positive or negative role for FSAP?

The accelerated atherosclerotic plaques in MI-carriers can be explained by the missing inhibitory effect of FSAP on PDGF-BB cleavage as well as a possible effect on the endovascular uPA system. In this study, these mechanisms were identified as the key players of FSAP action in an *in vivo* model of vascular remodeling.

On the other hand, a stable fibrous cap can protect the plaques from rupture[29]. FSAP staining was predominantly found in macrophage rich areas of instable atherosclerotic lesions in co-localization with uPA[75]. The inhibitory effect of FSAP on VSMC could account for a thin muscular layer, thus making the plaque susceptible for rupture. The observed FSAP induced activation of MMP-2/9 can additionally destabilize the plaque and explain the clinical findings.

Interestingly, there are parallels between the FSAP treated vessels and the time course experiment after irradiation and BMTX with respect to the stability of the neointimal lesion. The FSAP treated vessels at 4 weeks after dilatation looked very similar to the buffer control treated vessels at 2 weeks after injury. Thus, the FSAP treated vessels seem to maintain an earlier developing status due to a lack of proliferation/ migration of resident VSMC. The stability of a plaque or the neointima is generally dependent on VSMC secreting ECM proteins and forming a fibrous cap. Since the neointima of FSAP treated arteries without a previous irradiation consists of regular VSMC, irradiation obviously has additional and deleterious effects on vascular remodeling[93]. However, the control arteries have been irradiated in the same way, and the inhibitory effect of FSAP on VSMC has also been demonstrated in a model without preceding irradiation, so that these two inhibitory effects probably worked in an additive way, thus leading to an instable appearence of the neointima.

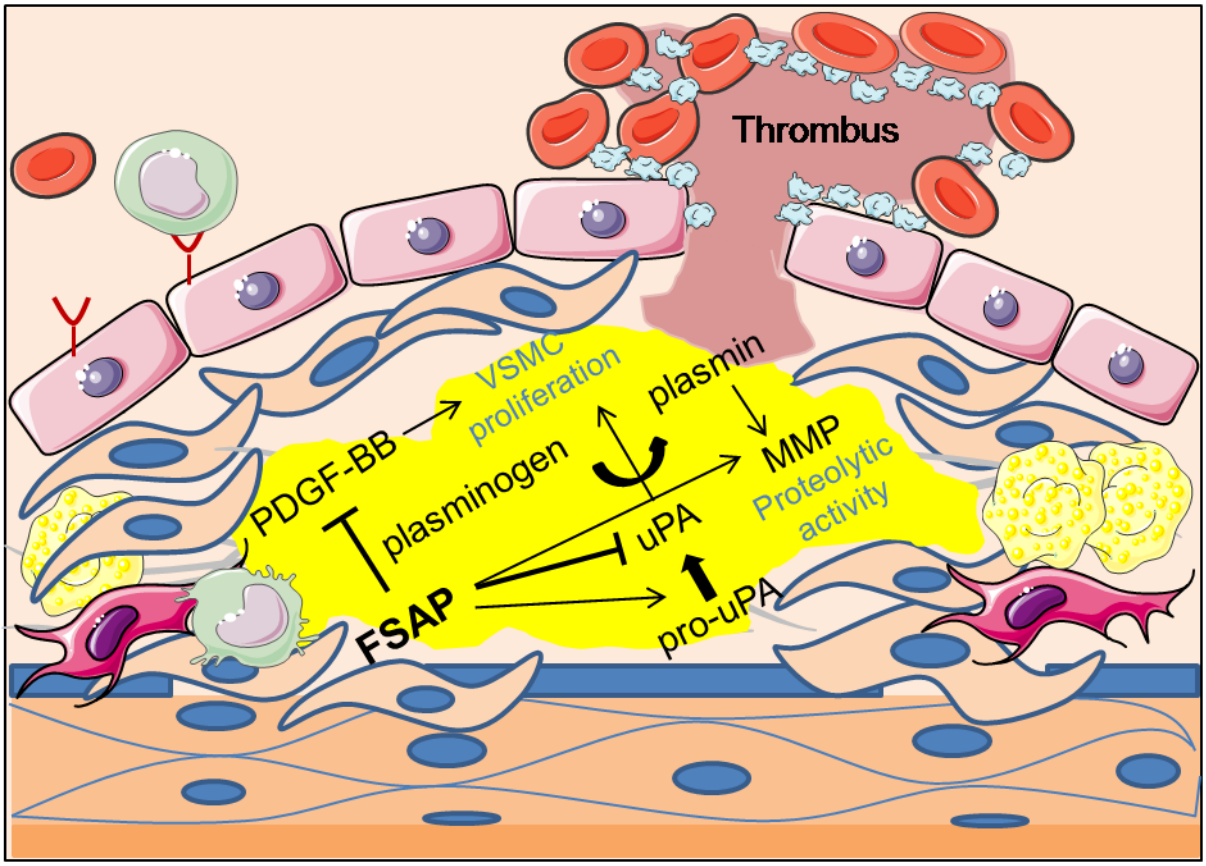

Figure 27. FSAP functions in vascular remodeling

FSAP can directly cleave PDGF-BB, which is an important growth factor vor VSMC proliferation. Moreover, FSAP decreases the activity of uPA over time and thus further inhibits the proliferation of VSMC. MMP are activated by uPA generated plasmin, however, the MMP activity was enhanced after FSAP treatment. Therfore, FSAP seems to have a direct effect on MMP activity. Hence, FSAP inhibits VSMC proliferation and regulates the protease balance at sites of tissue remodelling.

5.6 Perspective

In the presented work, we could provide evidence for an inhibitory effect of FSAP on VSMC by a specific inhibition of PDGF-BB signaling and a down-regulation of uPA *in vivo*. Additionally, there was no effect of FSAP on transdifferentiation of BMPC in the neointimal lesion.

Recently, we have generated FSAP$^{-/-}$ mice. The first dilatated mice showed a very pronounced neointima formation at 2 weeks after dilatation. Since FSAP inhibits VSMC proliferation, these results confirm our previous findings.

The aim of the future studies on FSAP will deal with other animal models to mimic the process of atherosclerosis more precisely. In addition to the wire induced injury model, a cuff model of atherosclerosis will be used[104]. Therefore, the FSAP$^{-/-}$ mice can be backcrossed with

ApoE$^{-/-}$ mice, or lipoproteins of FSAP$^{-/-}$ mice can be knocked down by intravenously injected siRNA. The latter experimental setting has been recently established in the laboratory of Molecular Cardiology in Giessen[105].

Since FSAP can now be recombinantly produced, we are no longer dependent on the use of FSAP isolated from human plasma for our future work (unpublished data). Furthermore, adenoviral vectors encoding the FSAP gene have been developed, so that FSAP can be overexpressed in WT mice or reconstituted in the FSAP$^{-/-}$ mice. Accordingly, we can either target the liver to achieve a higher FSAP concentration in the circulation by injecting the vector intravenously, or we can apply the vector in a thermosensitive pluronic F-127 gel directly to the artery. As confirmed with other adenoviral vectors, they can also be intraluminally injected either into the external carotid artery or into the femoral artery, while the more proximal side of the artery is transiently ligated to stop the blood flow for several minutes[106].

In the vascular wall, FSAP is predominantly expressed in macrophages of instable atherosclerotic plaques. Although there was virtually no difference in the infiltration of monocytes/ macrophages after dilatation, it remains elusive, whether an uptake of FSAP by monocytic cells can possibly influence the regulation of adhesion molecules. Recent *in vitro* experiments performed in the Institute of Biochemistry showed that FSAP can up-regulate adhesion molecules, e. g. VCAM-1, in monocytes and therefore possibly promote their effect in atherosclerosis. However, we need to test these effects in more specific models of leucocyte adhesion, but in regard to the reduction of neointima formation after FSAP treatment, our *in vivo* experiments do not favor this effect to be of essential importance.

Interestingly, Wasmuth et al. could recently show that in patients with the MI-SNP the risk for HCV-induced liver fibrosis and cirrhosis is significantly increased[107]. They explain this effect probably due to the impaired PDGF-BB-mediated hepatic stellate cell proliferation by MI-FSAP.

With ~5% of heterozygous carriers of the MI polymorphism in the European population, the various effects of FSAP, either the good or the bad, are of fundamental interest for the care of millions of people. Although we were able to shed more light on the *in vivo* effects of FSAP in the vasculature, this work was just the beginning of more complex and refined methodical studies in the future. Therefore, it is indispensable that the molecular and clinical research complement one another in finding the best strategy to elucidate the wide range of FSAP functions in the human body.

6. Summary

Factor VII activating protease (FSAP), a novel plasma protease, can activate both Factor VII independently of tissue factor and pro-uPA. The FSAP gene has been linked to vascular diseases in humans, since the Marburg I (MI, G534E) polymorphism is a prominent risk factor for atherosclerosis and stroke[4, 5]. Furthermore, enhanced FSAP staining was detected in instable atherosclerotic plaques. In contrast to WT-FSAP, MI-FSAP has lower enzymatic activity and does not inhibit proliferation of vascular smooth muscle cells (VSMC) due to specific cleavage of platelet-derived growth factor (PDGF)-BB *in vitro*.

The effect of WT- and MI-FSAP on neointima formation was investigated in a mouse model of wire induced injury of the femoral artery. WT-FSAP was locally applied to the denuded artery in different concentrations and was then compared to MI-FSAP, as well as the active site-inhibited Phe-Pro-Arg-chloromethylketone (PPACK)-FSAP, and a buffer control. WT-FSAP attenuated neointima formation in a dose dependent manner and inhibited proliferation of VSMC, as determined by PCNA expression. Since MI-FSAP and PPACK-FSAP did not attenuate neointima formation, the effects of FSAP were mainly due to its proteolytic activity. Following *in situ* zymography, application of WT-FSAP changed the proteolysis balance in the vessel wall by reducing endogenous plasmin activity. Corresponding to this regulation, the neointima in $uPA^{-/-}$ mice was mainly acellular and nearly completely lacked VSMC. Furthermore, FSAP application did not influence the transdifferentiation of bone marrow derived progenitor cells into VSMC. Indeed, these cells were predominantly identified as macrophages and could no longer be detected in the vascular wall, when the inflammatory response to the vascular injury had calmed down.

The inability of MI-FSAP to inhibit VSMC proliferation *in vivo* explains the observed linkage between the MI-polymorphism and increased cardiovascular risk. Moreover, FSAP is a prominent regulator of the proteolysis balance at sites of tissue remodelling and could thus account for its association with plaque stability. Hence, FSAP is an important regulator of vascular remodelling with high clinical relevance.

7. Exposée

Die Faktor VII aktivierende Protease (FSAP) ist ein Plasmaprotein, das sowohl Faktor VII als auch pro-uPA aktiviert[86]. Der Marburg I (MI, G534E) Polymorphismus des FSAP-Gens gilt als bedeutender Risikofaktor für Atherosklerose[4, 5]. FSAP konnte in einer klinischen Studie in instabilen atherosklerotischen Plaques nachgewiesen werden[75]. MI-FSAP zeigte eine reduzierte proteolytische Aktivität und inhibiert im Gegensatz zu WT-FSAP nicht die Proliferation von glatten Gefäßmuskelzellen durch eine spezifische Spaltung von Platelet-derived growth factor-BB (PDGF-BB) *in vitro*[79].

In einem Mausmodel wurde durch Dilatation der A. femoralis eine Neointimaentwicklung induziert. Anschließend wurden verschiedene Konzentrationen von WT-FSAP um die Arterie gegeben, und diese mit MI-FSAP, dem am aktiven Zentrum inhibierten Phe-Pro-Arg-chloromethyketone (PPACK-) FSAP oder einer Kontrolle ohne FSAP verglichen. Die Applikation von WT-FSAP verminderte konzentrationsabhängig die Neoinitmabildung und inhibierte die Proliferation glatter Gefäßmuskelzellen, während MI-FSAP nur einen marginalen und PPACK-FSAP keinen Effekt hatte. Weiterhin konnten wir in einer *in situ* Zymographie zeigen, dass die Expression von aktivem uPA im Zeitverlauf nach Dialtation durch Applikation von WT-FSAP herunterreguliert wurde, was ebenfalls die Proliferation und Migration von glatten Gefäßmuskelzellen hemmte. In diesem Zusammenhang zeigte sich auch ein Fehlen von glatten Gefäßmuskelzellen in der Neointima von $uPA^{-/-}$ Mäusen. Weiterhin hatte FSAP keinen Einfluss auf die Transdifferenzierung von aus dem Knochenmark stammenden Progenitorzellen in glatte Gefäßmuskelzellen. Bei diesen Zellen handelte es sich fast ausschließlich um Makrophagen, die langfristig im vaskulären Remodeling nicht mehr nachweisbar waren.

In einem *in vivo* Model zeigte sich eine inhibierende Wirkung von WT-FSAP auf glatte Gefäßmuskelzellen und eine Beeinflussung der perizellulären Proteolyse. Durch die vermehrte Atherosklerose in Trägern des MI-Polymorphismus sowie die Färbung von FSAP insbesondere in instabilen Plaques lassen sich die Ergebnisse direkt auf die klinische Situation übertragen und unterstreichen die Wichtigkeit von FSAP im vaskulären Remodeling.

8. References

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9. Acronyms and abbreviations

α-sma	α-smooth muscle actin
α-sma$^+$	α-smooth muscle actin positive
µg	microgram
µM	micromolar
ADP	adenosine diphosphate
ApoE	apolipoprotein E
AT	antithrombin
bFGF	basic fibroplast growth factor
BM	bone-marrow
BMPC	bone-marrow derived progenitor cells
BMTx	bone-marrow transplantation
BSA	bovine serum albumin
CCR2	CC motif receptor-2
CD	cluster of differentiation
CRP	C-reactive protein
d	day
DAPI	4',6-diamidino-2-phenylindole
D-MEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DQ	dye quenched
EC	endothelial cells
ECM	extracellular matrix
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
ERK	extracellular signal-regulated kinases
F	factor
FCS	fetal calve serum
fig	figure
FSAP	factor seven activating protease
Glu	glutamic acid
Gly	glycine
GPI	glycosylphosphatidylinositol

Gy	Grey
h	hour
H_2Odd	aqua ad iniectabilia
H & E	hematoxylin & eosin
HGF	hepatocyte growth factor
ICAM	inter-cellular adhesion molecule
IFN-γ	interferon-γ
IGF	insulin-like growth factor
IL	interleukin-1
LDL	low-density lipoprotein
LRP	lipoprotein receptor-related protein
MAPK	mitogen-activated protein kinases
MCP	monocyte chemoattractant protein
M-CSF	macrophage-colony stimulation factor
MHC	major-histocompatibility complex
MI-FSAP	Marburg I-FSAP
min	minute
ml	millilitre
mM	milimolar
MMP	matrix metallo proteinases
MPI	Max-Planck-Institute
mRNA	messanger ribonucleic acid
n	number
NaCl	sodium chloride
n. s.	not significant
NI	neointima
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PC	protein C
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PECAM	platelet/endothelial cell adhesion molecule
PFA	paraformaldehyde

PHBP	plasma hyaluronan-binding protein
PN	protease nexin
PPACK-FSAP	Phe-Pro-Arg-chloromethyketone-FSAP
PS	protein S
PTA	percutaneous transluminal angioplasty
PTCA	percutaneous transluminal coronar angioplasty
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
Sca	stem cell antigen
SD	standard deviation
SDF	stromal cell-derived factor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	single-nucleotide polymorphism
TF	tissue factor; factor III
TFPI	tissue factor pathway inhibitor
TGF	transforming growth factor
Th1	type 1 helper T cell
TIMP	tissue inhibitors of metalloproteinases
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
tPA	tissue type plasminogen activator
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
uPA	urokinase type plasminogen activator
uPAR	urokinase type plasminogen activator receptor
VCAM	vascular cell adhesion molecule
VSMC	vascular smmoth muscle cells
vWF	von-Willebrand factor
w	week
WT-FSAP	wild-tipe-FSAP
WT-mice	wild type mice

10. Publications

10.1 Articles

1. **Factor VII activating protease (FSAP); a key regulator of pericellular proteolysis.**
   Daniel JM, Hersemeyer K, Muhl L, Tillmanns H, Preissner KT, Sedding D, Kanse SM.
   In preparation

2. **Time course analysis on the differentiation of bone marrow-derived progenitor cells into smooth muscle cells during neointima formation.**
   Daniel JM, Bielenberg W, Stieger S, Weinert S, Tillmanns H, Sedding, D.
   Arterioscler Thromb Vasc Biol. 2010 Oct;30(10):1890-6.

3. **A key role for Toll-like receptor-3 in disrupting the hemostasis balance on endothelial cells.**
   Shibamiya A, Hersemeyer K, Schmidt Wöll T, Sedding D, Daniel JM, Bauer S, Koyama T, Preissner KT, Kanse SM.
   Blood. 2009 Jan 15;113(3):714-22.

4. **Urokinase receptor (CD87) clustering in detergent-insoluble adhesion patches leads to cell adhesion independently of integrins.**
   Petzinger J, Saltel F, Hersemeyer K, Daniel JM, Preissner KT, S, Wehrle-Haller B, Kanse SM.
   Cell Commun Adhes. 2007 Oct;14(4):137-55.

5. **The G534E polymorphism of the gene encoding the factor VII-activating protease is associated with cardiovascular risk due to increased neointima formation.**
   Sedding D, Daniel JM, Muhl L, Hersemeyer K, Brunsch H, Kemkes-Matthes B, Braun-Dullaeus RC, Tillmanns H, Weimer T, Preissner KT, Kanse SM.
   J Exp Med. 2006 Dec 25;203(13):2801-7.

10.2 Abstracts

1. Time course analysis of bone marrow derived progenitor cell transdifferentiation during neointima formation

J.-M. Daniel, H.H. Tillmanns, D.G. Sedding

Accepted as an oral presentation at the XXXI Congress of the European Society of Cardiology, 29th august – 4th September 2009, Barcelona, Spain

2. Factor VII activating protease (FSAP) alters the pericellular proteolysis balance in the vessel wall during neointima formation

J.-M. Daniel, K. Hersemeyer, O. Uslu , O. Rannou, L. Muhl, K. T. Preissner, D.G. Sedding, S.M. Kanse

Accepted as a poster presentation at the XXXI Congress of the European Society of Cardiology, 29th August – 4th September 2009, Barcelona, Spain

3. Factor VII activating protease (FSAP); a key regulator of pericellular proteolysis

J.-M. Daniel, O. Uslu, K. Hersemeyer, O. Rannou, L. Muhl, K. T. Preissner, D.G. Sedding, S.M. Kanse

Accepted as an oral presentation at the XXII Congress of the International Society on Thrombosis and Haemostasis, 11th July – 16th July 2009, Boston, U.S.A

4. Time course analysis of bone marrow derived progenitor cell transdifferentiation during neointima formation

J.-M. Daniel, H.H. Tillmanns, D.G. Sedding

Oral presentation, 75. Jahrestagung der Deutschen Gesellschaft für Kardiologie – Herz- und Kreislaufforschung, Mannheim, 2009

5. Time course analysis of bone marrow derived progenitor cell transdifferentiation during neointima formation

J.-M. Daniel, H.H. Tillmanns, D.G. Sedding

Poster presentation, 88. Jahrestagung der Deutschen Physiologischen Gesellschaft, Giessen, 2009

6. Role of Sirt1 in Vascular Homeostasis and Pathologic Remodelling

D.G. Sedding, S. Vogel, J.-M. Daniel, H. Koenig, H.H. Tillmanns

75. Jahrestagung der Deutschen Gesellschaft für Kardiologie – Herz – und Kreislaufforschung, Mannheim, 2009

1. Rudi Busse Young Investigator Award

7. Sirt1 hemmt die Apoptose Glatter Gefäßmuskelzellen

D.G. Sedding, S. Vogel, H. Koenig, J.-M. Daniel, H.H. Tillmanns

74. Jahrestagung der Deutschen Gesellschaft für Kardiologie – Herz – und Kreislaufforschung, Mannheim, 2008

8. Sirt1 Reguliert die Funktion Glatter Gefäßmuskelzellen und das Vaskuläre Remodeling

S. Vogel, H. Koenig, J.-M. Daniel, H.H. Tillmanns, D.G. Sedding

73. Jahrestagung der Deutschen Gesellschaft für Kardiologie – Herz – und Kreislaufforschung, Mannheim, 2007

9. Sirt1 Reguliert die Funktion Glatter Gefäßmuskelzellen und das Vaskuläre Remodeling

S. Vogel, H. Koenig, J.-M. Daniel, H.H. Tillmanns, D.G. Sedding

73. Jahrestagung der Deutschen Gesellschaft für Kardiologie – Herz – und Kreislaufforschung, Mannheim, 2007

10. Der G511E Polymorphismus Führt zum Verlust der Antiproliferativen Aktivität der Faktor VII aktivierenden Protease (FSAP) und begünstigt die Entstehung Vaskuloproliferativer Erkrankungen

D. Sedding, J.-M. Daniel, L. Muhl, K. Hersemeyer, H. Brunsch, R. Braun-Dullaeus, H. H. Tillmanns, K. T. Preissner, S. M. Kanse

72. Jahrestagung der Deutschen Gesellschaft für Kardiologie – Herz – und Kreislaufforschung, Mannheim, 2006

2. Rudolf-Thauer-Posterpreis

11. Urokinase Receptor (uPAR)(CD87) – Clustering In Detergent Insoluble Adhesion Patches (DIAP) Leads To Cell Adhesion On A Vitronectin Matrix

J.-M. Daniel, J. Petzinger, K. Hersemeyer, K. T. Preissner, S. M. Kanse

Poster presentation at the XXII Congress of the International Society on Thrombosis and Haemostasis, 4th July – 12th August 2005, Sydney, Australia

12. The association of factor VII activating protease (FSAP) polymorphism (G511E) with atherosclerosis is due to its diminished ability to inhibit cell proliferation.

D. Sedding, J.-M. Daniel, L. Muhl, K. Hersemeyer, H. Brunsch, B. Kemkes-Matthes, R. Braun-Dullaeus, H.H. Tillmanns, T. Weimer, K. T. Preissner, S. M. Kanse

Poster presentation at the XXII Congress of the International Society on Thrombosis and Haemostasis, 4th July – 12th August 2005, Sydney, Australia

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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Erklärung

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht.

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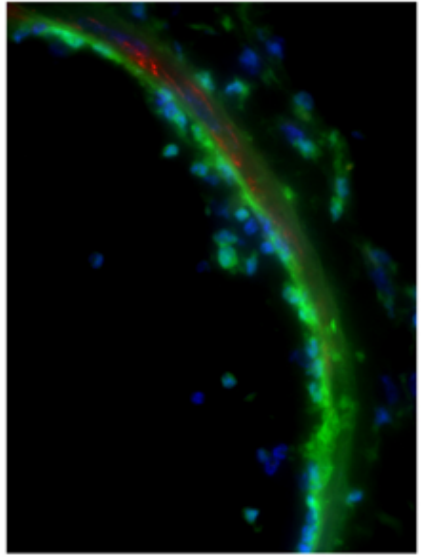

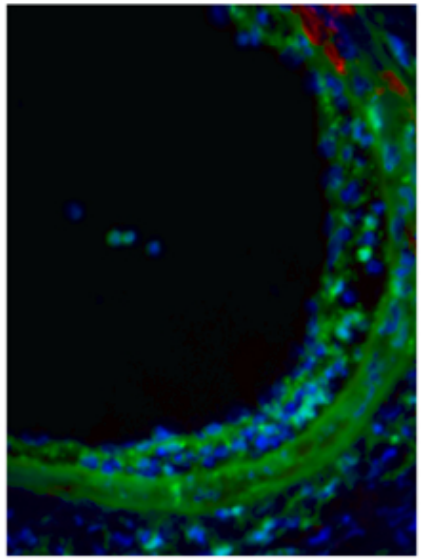

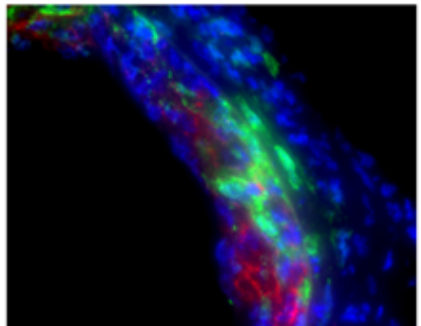

The in vivo effects of FSAP on neointima formation

Jan-Marcus Daniel